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Production of Bioethanol by Fermentation of Sugars Released by Dilute-acid and Enzymatic Hydrolysis of Soybean Meal

Deivis E. Lujan-Rhenals
University of Arkansas, Fayetteville

PRODUCTION OF BIOETHANOL BY FERMENTATION OF SUGARS RELEASED BY DILUTE-ACID AND ENZYMATIC HYDROLYSIS OF SOYBEAN MEAL

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A dissertation submitted in partial fulfillment
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By

Deivis Enrique Luján Rhenals
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ABSTRACT

Soybean meal (SBM) is a co-product of soybean oil extraction mostly used as animal feed due to its protein content ranging from 40 to 49%. Additionally, SBM contains 35-42% of carbohydrates, half of which are structural, and the remaining consists of approximately 17% of mono- and disaccharides—especially sucrose—up to 8% oligosaccharides, and 1-2% starch. When used as animal feed, only sucrose and starch are digested and absorbed by monogastric animals. Although SBM contributes carbohydrates to their diet, its main function is to provide proteins. Therefore, the selective removal of carbohydrates would create a protein-enriched meal with a greater value, which would facilitate the formulation of diets, and a byproduct stream rich on fermentable sugars that could be used as a feedstock for fermentations. The aim of this research was to develop a process to treat SBM with a combination of treatments with dilute sulfuric acid at different concentrations, temperatures, and times followed by enzymatic hydrolysis with cellulase and ß-glucosidase after detoxification with activated carbon to reduce inhibitor effects. The final product was a high-protein SBM and a liquid fraction rich in fermentable sugars that was used in the production of ethanol via fermentation with *Saccharomyces cerevisiae* and *Zymomonas mobilis*. Treatments enhanced the crude protein content up to 58.6% d.b. with a lysine bioavailability up to 97%. An important balance among fermentable sugars (16.2% d.b.), crude protein (55.5% d.b.) and color (close to untreated SBM) was reached with the treatment at 120°C, 1.5% H_2SO_4, and 30 min. *S. cerevisiae* yielded its maximum bioethanol production at 8 g/L and *Z. mobilis* 9.2 g/L without any supplementation of the fermentation broth.

This dissertation is approved for recommendation
to the Graduate Council

Dissertation Director

_____

Dr. Rubén O. Morawicki

Dissertation Committee:

_____

Dr. Jean-François Meullenet

_____

Dr. Ya-Jane Wang

_____

Dr. Steven C. Ricke

_____

Dr. Edward E. Gbur

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DEDICATION

To Carmen Eladia and David Manuel

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CHAPTER I

Introduction and Research Objectives

In recent years, alternative energy sources are becoming more crucial and bioethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels (Friedl, 2012, Cardona and Sánchez, 2007, Lin and Tanaka, 2006). However, for bioethanol to be competitive with traditional sources of fossil fuels, it is important to reduce the costs associated with its production (Del Campo et al., 2006). At present, many starch containing crops, such as corn and wheat, and crops containing fermentable sugars, e.g. sugarcane, are being used to produce bioethanol by fermentation, but it is necessary to look for other raw materials in order to: (1) reduce environmental problems, (2) eliminate the competition between the food and energy sectors for agricultural crops, and (3) maximize cost efficiency (Tengborg et al., 1998). As a result, other non-food crops, such as lignocellulosic and hemicellulosic materials, are being evaluated for their potential use in the production of bioethanol. The trend in fuel ethanol production is to reduce the cost of feedstock through the exploitation of less expensive lignocellulosic biomass and other by-products. On the whole, researchers are interested not only in the production of fermentable sugars from lignocellulosic materials, but also in the generation of useful intermediates (Cardona and Sánchez, 2007).

By-products and co-products of the food industry could be considered for this purpose, especially when there is a significant amount of carbohydrates with no defined application that could be extracted and used for the production of bioethanol (Edwards and Doran-Peterson, 2012). Soy bean meal (SBM), an abundant co-product of the soybean oil industry, is predominantly used as the main protein source in animal feed. But SBM also contain a

carbohydrates portion that could be used for a better purpose. Hypothetically, SBM could be even more profitable if the carbohydrates are extracted and used in the manufacture of more valuable products (e.g. bioethanol) before animal consumption, which would create at the same time an enhanced protein SBM.

Compared to other sources of protein for animal feed, SBM has the highest protein quality with an excellent amino acid composition (Cromwell, 1999) and overall nutrient content. It is low in fiber and is the most energy-dense among the plant protein sources used for feed stock (Waldroup, 2006). In addition to the nutritional advantages, SBM is easily obtained compared to several of the other alternative protein sources (Lim et al., 2004; Hardy, 1996). It is the most economical protein source available on the market and has the most consistent feed ingredients available to the feed manufacturer (Smith, 2010).

In addition to proteins, SBM contains 40-42 % d.b. carbohydrates (Da Silva et al., 2009; Karr-Lilienthal et al., 2005b). These carbohydrates could be removed selectively and used as a substrate for the industrial production of compounds such as bioethanol, organic acids, and microbial biomass. The reduction in carbohydrate content would potentially generate a protein-enriched SBM product with improved amino acid bioavailability with other potential applications (e.g. aquiculture, swine, and poultry).

There are some reports in the literature about the use of SBM carbohydrates for ethanol production. Siqueira et al. (2008) demonstrated that soybean molasses—a byproduct of the production of soybean concentrate after aqueous alcohol extraction— can be used as a substrate for the production of bioethanol without supplementation or pH adjustment, since soybean molasses provided the necessary carbohydrates, nitrogen, magnesium and the appropriate

hydrogen balance for the fermentation with *S. cerevisiae*. Similarly, Letti et al. (2012) proved the feasibility of bioethanol production from soybean molasses by *Z. mobilis*.

A different approach to the one taken by Siqueira et al. (2008) and Letti et al. (2012) would be the use of dilute acid and enzymatic hydrolysis on SBM that would target the carbohydrate portion. The hypothesis of this research is that these treatments would remove carbohydrates from the SBM and create a protein-rich SBM and a byproduct with high content of fermentable sugars. Also, it is expected that the treatments will improve protein bioavailability, which would benefit SBM producers and the feed industry, the main consumer of SBM in the US. (USDA, 2012)

The overarching goal of this research is to treat soybean meal with dilute acid and enzymes at different conditions to extract fermentable sugars, use these sugars as substrates for the production of ethanol; and, in the process, to create a SMB protein with enhanced properties. Specific objectives are as follows:

Objective 1: To determine the optimum acid hydrolysis conditions at atmospheric pressure to produce fermentable sugars from soybean meal while enhancing its protein content (Chapter 3)

Objective 2: To establish the optimum acid hydrolysis conditions under high pressure to obtain fermentable sugars from soybean meal while enhancing its protein content (Chapter 4).

Objective 3: To evaluate the enzymatic hydrolysis of acid-hydrolyzed soybean meal using Cellulase, ß-glucosidase, and a mix of cellulase with β-glucosidase and assess Lysine bioavailability after the enzymatic treatments (Chapters 5 and 6).

Objective 4: To evaluate the bioethanol production from the liquid fraction of acid- and enzyme-hydrolyzed soybean meal by batch fermentation with *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Chapter 7).

CHAPTER II

Literature Review

A. Soybean meal

SBM is obtained from cleaned whole soybeans generally following this process: tempering, cracking and dehulling, flaking, solvent extraction, flash desolventizing, toasting, drying, cooling, milling, and classification (Erickson, 1995). A typical flow diagram of SBM production is shown in Figure 2.1 (Kumar et al., 2002).

1. Composition of soybean meal

Da Silva et al. (2009) reported that SBM is composed of 89.5% dry matter including 17% neutral detergent fiber, 6.97% acid detergent fiber, and 42.02% total carbohydrates. Baker et al. (2009) reported that new varieties can have high protein and lysine content of 54.86% and 3.56%, respectively. A large fraction of the polysaccharides present in SBM is cellulose and more than half of the polysaccharides are pectic substances (Fischer et al., 2001). Karr-Lilienthal et al. (2005b) reported that of the 40% d.b. carbohydrates in SBM, approximately half are non-structural in nature—low-molecular weight (LMW) sugars, oligosaccharides, and starch in small quantities—and the other half are structural polysaccharides (with a large amount of pectic polysaccharides). Similarly, Grieshop et al. (2003) indicated that the total non-structural carbohydrate (TNC) content is 13.6-17.9% d.b with low molecular weight sugars (such as glucose, arabinose, galactose, fructose, and sucrose making up the majority (17% d.b.); thus, LMW sugars constitute nearly 50% of the total carbohydrates. The main oligosaccharides are galacto-oligosaccharides (such as stachyose, raffinose and verbascose in small amounts) and they represent 4-8% d.b. of the total SBM mass (Table 2.1). Starch is present in low concentrations (approximately 1% d.b.). The structural polysaccharides are the remaining carbohydrates in SBM

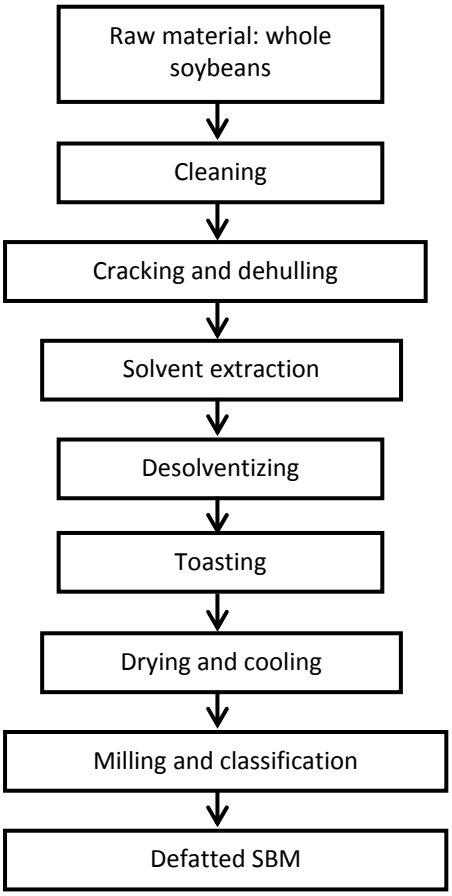


Figure 2.1. Soybean meal production (Kumar et al., 2002, Erickson, 1995)

and they include dietary fiber (cellulose, pectin, and hemicelluloses), mannans, galactans, and xyloglucans. However, the structural polysaccharides in SBM are highly varied, and the specific structures are not well understood. Ultimately, due to the high concentrations of pectic and cellulosic material, for the carbohydrates to be utilized in fermentation, the SBM must be subjected to hydrolysis—chemical or enzymatic.

Smith (2010) reported that the typical composition of SBM available to the U.S. feed manufacturer is 44% crude protein, 0.5% ether fiber, 7% crude fiber, and 6.0% ash, with 2240 Kcal/kg metabolizable energy for poultry. The amino acid composition of SBM (with concentration expressed in percent d.b.) is: arginine (3.4), lysine (2.9), methionine (0.65), cysteine (0.67), tryptophan (0.6), histidine (1.1), leucine (3.4), isoleucine (2.5), phenylalanine (2.2), threonine (1.7), and valine (2.4). However, Karr-Lilienthal et al. (2005a) found that the composition of SBMs from 55 commercial U.S. processing plants differed in composition and quality due to differences in processing conditions such as moisture, drying time, and toasting or drying temperature.

Table 2.1.Oligosaccharide and polysaccharide concentrations of dehulled soybean meal (SBM)[a]

Component	**% of SBM db**
Oligosaccharides, total	15
Sucrose	6–8
Stachyose	4–5
Raffinose	1–2
Verbascose	Trace
Polysaccharides, total	15-18
Acidic polysaccharides	8-10
Arabinogalactan	5
Cellulosic material	1-2
Starch	0.5

[a] Honig and Rackis (1979).

2. Production of soybean meal

Globally, the 2012 August projection in soybean production for 2012-13 is 260.46 million metric tons, while in the USA the soybean production is projected at 73.27 million metric tons, which represent 28.1% of the world soybean production (USDA, 2012). The soybean meal (SBM) show similar data; for instance, it is projected that the world production for the period 2012/2013 is projected at 179.19 million metric tons; the projection for the United States is 32.66 million metric tons (18.2% of the world's production) followed by Argentina and Brazil with projected SBM productions of 29.8 million metric tons (16.6% of the world's production) and 28.52 million metric tons (15.9%), respectively. The SBM production in the United States has increased steadily from 24 million tons in 1980 to 40 million tons in 2008. In August, 2012-2013, the SBM projected price was $460-490 per short ton, while the month before was $125 to 146 per short ton (USDA, 2012).

3. Uses of soybean meal

SBM is the predominant source of protein in the animal feed industry and the most economical protein source available on the market (Smith, 2010). The total SBM use in the U.S. is estimated to be 26.58 million tons for 2012-2013 (USDA, 2012). SBM is currently used for: poultry feed (48%), swine feed (26%), bovine feed (12%), dairy products (9%), pet foods (3%), and other applications (2%) (Soystats.com, 2012).

SBM is considered one of the most suitable and stable protein sources for fish and could be used as a fish meal replacement for commercial freshwater species, such as carp, tilapia, and catfish (Kikuchi, 1999). SBM is also used for feedstock and for the production of soy protein isolate; there is further potential use in the animal feed industry with enzymatic modification of

the polysaccharides in SBM since monogastric animals cannot take advantage of the polysaccharides present in native SBM (Huisman et al. 1998).

Many poultry nutritionists prefer dehulled SBM over SBM with only 44% protein due to its higher energy content, balanced protein, and lower fiber level; besides, due to the greater protein content, the proportion of cereal grain in the diet could be augmented with SBM and the extra energy could improve weight gains and reduce the amount of feed required per unit gain (Smith, 2010).

B. Acid hydrolysis of lignocellulosic materials

Acid hydrolysis is a very common and effective treatment method to modify the structure of lignocellulosic materials. Chemical hydrolysis (acid or alkaline) requires treatment for a period of time at a particular temperature in order to generate monomeric sugars from lignocellulosic material. Sulfuric acid is the most common chemical used for chemical hydrolysis (Harris et al., 1945). Usually, acid hydrolysis is divided into two categories: concentrated acid hydrolysis and dilute acid hydrolysis. The former has the advantages of low operating temperatures with high sugar yields. The disadvantages of concentrated acid, however, include significant acid consumption, equipment corrosion, acid recoveries that require significant energy, and longer reaction times. The dilute acid process, in contrast, utilizes significantly less acid and requires relatively short residence times, but it does require higher temperatures; however, there is lower sugar yield, equipment corrosion, and formation of undesirable by-products (Taherzadeh and Karimi, 2007).

In spite of its disadvantages, dilute acid hydrolysis is the most commonly used method of chemical hydrolysis. It is used as a pretreatment for enzymatic hydrolysis or to directly hydrolyze lignocellulosic material to sugars (Qureshi and Manderson, 1995). Dilute acid

hydrolysis of lignocellulosic material can be a one- or two-stage process. In two-stage hydrolysis, the first stage involves the depolymerization of hemicellulose by treating the sample with dilute acid at 140ºC for 15 min. The lower temperature treatment of hemicellulose is carried out to avoid the formation of furan compounds and carboxylic acids. In the second stage, the sample is held at 190ºC for 10 min to hydrolyze cellulose. A treatment at lower temperatures (121ºC) can further reduce the formation of furfural and 5-hydroxymethylfurfural (5-HMF) but sugar yields are lower (Saha et al., 2005a, b).

At temperatures less than 200ºC, most of the hemicellulose (more than 80%) can be hydrolyzed by dilute acid hydrolysis; however, due to the recalcitrance of cellulose to dilute acid hydrolysis, maximum glucose yields are only attainable with temperatures greater than 220ºC; however, the most important issue in one-stage dilute acid hydrolysis is the degradation of sugars and the formation of undesirable by-products. These by-products reduce sugar yields and also inhibit ethanol production during fermentation. Furfural, 5-HMF, acetic acid, phenol, levulinic acid, uronic acid, 4-hydroxybenzoic acid, vanillic acid, vanillin, cinnamaldehyde, and formaldehyde are among the most common inhibitors produced in this kind of reaction (Taherzadeh, 1999; Lee et al., 1999; Larsson et al. 2000). At temperatures less than 160ºC, hemicellulose hydrolysis is not homogeneous because one fraction can be rapidly hydrolyzed whereas the remainder is hydrolyzed slowly (Lee and Lyer, 1999).

Normally, an acid pretreatment step is necessary to modify the structural characteristics of lignocellulosic material because glucan and xylan are more susceptible to enzymes following the pretreatment (Kumar and Wyman, 2009). Hydrolysis at higher temperatures and pressures could reduce the reaction times and their effects on the sugars (Taherzadeh and Karimi, 2007). The products of hemicellulose hydrolysis are pentoses and hexoses and the product of cellulose

hydrolysis is glucose. Glucose and fructose are the fermentable sugars at the highest concentration in the majority of acid treatments and are likely generated as a result of the breakdown of sucrose, stachyose, and raffinose as well as polysaccharides (cellulose and hemicellulose) originally present in the SBM, according to Montilla et al. (2009) and Iloukhani et al. (2001).

C. Detoxification after hydrolysis

During acid hydrolysis toxic compounds could be formed in different quantities depending upon the process conditions which need to be removed before fermentation in order to avoid microbial inhibition. For instance, previous research showed that bioethanol production with *Z. mobilis* is lowered by 20-40% when the 5-HMF concentration in the fermentation broth is above 0.09 g/L (Pienkos and Zhang, 2009). *S. cerevisiae*, on the other hand, is less susceptible to 5-HMF; for example, to reduce the production of bioethanol by 50%, the concentration of 5-HMF in the fermentation broth has to be around 8 g/L (Clark and Mackie, 1984); however, Taherzadeh et al. (1997) compared and correlated the performance of *S. cerevisiae* in lignocellulosic hydrolyzates to the content of acetic acid, formic acid, furfural, 5-HMF and phenol monomers and demonstrated that poor fermentability of dilute acid wood hydrolyzates by *S. cerevisiae* was correlated to high concentrations of furfural, 5-HMF and acetic acid.

Several methods that totally or partially eliminate these compounds have been studied (including chemical, physical, and biological methods) (Lee et al., 1999; Buhner and Agblevor, 2004; Carvalheiro, et al., 2005). Activated carbon is extensively used to remove these toxic compounds from the liquid fraction after acid hydrolysis; however, the efficiency of activated carbon depends upon the pH, contact time, temperature, and concentration (Mussatto and Roberto, 2004). Lee et al. (1999) studied the effect of charcoal treatments (compared to overlime

and silicate) on bioethanol fermentation in the range of 0.05-0.2 g charcoal/g glucose and they determined that detoxification with charcoal was more effective than the other methods tested.

In addition, detoxification is necessary to avoid enzyme inhibition (Szengyel and Zacchi, 2000). They found that the activity of both cellulase and ß-glucosidase decreased when the concentration of furfural was increased from 0 to 1.2 g/L. Cellulase activity decreased from 1.32 to 0.73 FPU/mL and the ß-glucosidase activity decreased by 50%. Finally, a similar study was reported by Ximenes, et al. (2010) in which it was demonstrated that certain phenols formed after acid hydrolysis also inhibited cellulase and ß-glucosidase activity.

D. Enzymatic hydrolysis of lignocellulosic materials

Fermentable sugars are produced after enzymatic hydrolysis of lignocellulosic materials and these sugars can be used by yeast and some bacteria to produce bioethanol during fermentation (Sun and Cheng, 2002). Compared to acid or alkaline hydrolysis, enzymatic hydrolysis is less expensive because it is usually carried out under milder conditions (pH 4.8 and 45-50ºC) and does not cause corrosion (Duff and Murray, 1996). Additionally, the bioethanol yields are greater during fermentation than the yields following acid hydrolysis since no toxic compounds are formed during enzymatic hydrolysis; however, enzyme hydrolysis is much slower than acid hydrolysis and enzymatic hydrolysis of lignocellulosic material often requires a pretreatment (Zhang and Lynd, 2004). Pretreatment is the principal challenge in bioethanol production from lignocellulosic material because it is a complex matrix of cellulose and lignin linked by hemicellulose chains. In order to reduce the crystallinity and increase the fraction of amorphous cellulose to facilitate enzymatic activity, the matrix must be subjected to a pretreatment process where hydrolysis of cellulose can be greater than 90% when otherwise it is less than 20% (Lynd et al., 1999).

Cellulases are very specific enzymes that hydrolyze cellulose (Béguin and Aubert, 1994). Normally, cellulases are a mixture of several enzymes. There are three main groups of enzymes that participate in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC3.21.4.), (2) exoglucanase or cellobiohydrolase (CBH, 1,4-ß-D-glucan cellobiohydrolase, or EC 3.2.1.91.) and (3) ß-glucosidase (EC 3.2.1.21) (Coughlan and Ljungdahl, 1988). Endoglucanase attacks areas of low crystallinity within the cellulose fiber creating free chain-ends. Endonucleases quickly depolymerize cellulose, whereas cellobiohydrolases gradually depolymerize the polysaccharide. Cellobiohydrolases work on crystalline cellulose by removing cellobiose units from the free chain-ends while endoglucanases principally work on the amorphous fraction (Lynd et al., 2002). ß- glucosidase is a cellobiohydrolase that hydrolyzes cellobiose into two molecules of glucose. It is normally extracted from *Trichoderma reesei*; however, it has low activity. Additionally, cellobiohydrolases are inhibited by cellobiose. For this reason, it is necessary to add ß-glucosidase from other sources in order to complement the activity of the enzyme extracted from *T. reesei* (Kim, et al., 1998; Kumar and Wyman, 2009).

Overall, among the available sources of enzymes, microbial cellulolytic enzymes are the most commonly used for cellulose hydrolysis. *T. reesei* is the most common mold used for commercial enzyme production. This mold releases a mixture of cellulases with at least two cellobiohydrolases, five endoglucanases, ß-glucosidases, and hemicellulases (Zhang and Lynd, 2004).

E. Bioavailability of amino acids

Bioavailability of dietary amino acids is defined as the fraction of ingested dietary amino acid absorbed in a chemical form that renders these amino acids potentially suitable for metabolism or protein synthesis (Batterham, 1992; Lewis and Bayley, 1995). Evaluating the

amino acid availability in protein sources of animals is crucial due to the variability in the protein composition of these diet components; however, there is no direct measure of amino acids bioavailability (Erickson et al., 2000). Traditionally, measures of in-vivo digestibility have been used to estimate AA bioavailability (Sauer and Ozimek, 1986). Another traditional method, described by Batterham (1992), utilizes a slope-ratio assay to estimate the bioavailability where the response—whole body protein deposition (Batterham, 1992) or AA oxidation (Moehn et al., 2005)—is related to the AA intake, and the slope of the regression line is compared with that of animals fed a defined reference protein source. The ratio of the slope of the test feed ingredient to the slope of the reference protein represents the relative bioavailability of the AA in question. Unfortunately, these methods are costly, tedious and time-consuming (2 to 4 weeks). Additionally, they require special facilities with large amounts of raw materials, they cannot be applied to a mixture of feed ingredients, and they generate data with high standard errors (Gabert et al., 2001, Erickson et al., 2000).

At the present time, other methods are also available, such as AA digestibility and microbiological (biosensor) assays, which are more suitable for estimating AA bioavailability than the slope-ratio assay (Chalova et al., 2007, Stein et al., 2007). Among them, microbiological assays for amino acids bioavailability are more effective in terms of time, cost and variability (Erickson et al., 2000). Biosensors that use *Escherichia coli* are the most reliable assays for quantification of amino acids bioavailability (Tuffnell and Payne, 1985; Anantharaman et al., 1983) with high correlation (> 0.9) with respect to chemical method in the quantification of available lysine (Anantharaman et al., 1983) and a good predictor of lysine bioavailability in a variety of protein sources for animals (Tuffnell and Payne, 1985). Although the methods are rapid and commonly implemented today, chemical methods for the

determination of amino acid concentrations in feed ingredients including high performance liquid chromatography (HPLC) and gas chromatography (GC) are not necessarily the most suitable since they generate values that are greater than the actual amounts of amino acids utilized under physiological conditions (Kivi, 2000)

1. Lysine bioavailability analysis by biosensor

Biosensors consist of enzymes, antibiotics, or microorganisms (mainly bacteria due to rapid growth), which can physiologically or chemically interact with low concentrations of a compound of interest. Biosensors are very specific, sensitive, and flexible to use, and they do not require large and expensive instrumentation such as chemicals analyses (Chalova et al., 2009). Currently, the requirement for rapid analytical tools with high specificity for food and fermentation analysis is increasing and expanding so new biosensor assays are continually being developed for analysis of nutritional components, food additives and contaminants. For instance, a common application of bacterial biosensors is for the determination of sugars (Chalova et al., 2009).

Microbial methods for quantification of amino acid bioavailability are precise, easy, specific and economical. They include different assay microorganisms and are based on their nutritive requirements for the respective amino acid (Shockman, 1963). Since it is necessary to develop new methods with equal reliability but less complex and faster, Chalova et al. (2009) demonstrated that *Escherichia coli* is effective as a biosensor for the determination of bioavailable amino acids, such as lysine, in feed proteins. *E. coli* is the most highly investigated microorganism for amino acid bioavailability quantification because this bacterium offers several advantages over other microorganisms (Payne and Tuffnell, 1980). These advantages are: (1) it has the lowest doubling time among bacteria; (2) it is easy to growth with minimal nutritional

supplementation of the media; (3) the genetics are very well established and easily recognized; and (4) it can be easily manipulated to produce desired phenotypic responses. Additionally, *E. coli* is naturally found in the gut microflora of the majority of animals and humans with an absorption of amino acids and peptides very similar, which make this bacterium very functional as a biosensor microorganism for these substances (Ingraham et al., 1983).

A bacterium has to be an auxotroph for the analyte in order to be used as a test microorganism; for instance, it should be incapable of synthesizing the amino acid of interest. Thus, the cell growth of the auxotroph would be a direct function of the concentration of the compound evaluated (Gavin, 1957). Consequently, the amount of the amino acid in the medium could be determined by the extent of bacterial cell growth. All 20 amino acids can be synthesized by the wild type *E. coli* while growing in medium containing only a carbon source and inorganic salts. As a result, the wild type *E. coli* cannot be used directly for amino acid quantification. Therefore, multiple mutants of *E. coli* have been created by genetic manipulation and studied for the purpose of quantifying amino acid bioavailability (Neidhardt et al., 1990).

F. Bioethanol fermentation

The U.S. Department of Energy (2010a) indicated that "In 2007, the President set a goal of reducing gasoline usage in the United States by 20 percent in the next 10 years. To achieve this goal, 15% of the reduction will come from increasing the supply of alternative fuels, and the remaining 5% from making motor vehicles more energy efficient. Displacing 15% of the projected gasoline usage for 2017 will require a rapid expansion of the annual renewable fuel supply from about 5 billion gallons of corn grain bioethanol to about 35 billion gallons of alternative fuels from a variety of plant materials including grasses, woodchips, and agricultural wastes."

Currently, the production of renewable fuels, such as bioethanol, obtained from agricultural residues is gaining in importance. Even though a large volume of this fuel is produced from sugar cane sucrose, bioethanol production from alternative sources can be attractive, especially when produced as a co-product associated with existing industries (Neureiter, et al., 2002). Biothanol produced from cellulosic material is considered a renewable option that may improve the local production of fuels, reactivate rural economics, and reduce pollution. According to U.S. Secretary of Energy Steven Chu, "Developing the next generation of biofuels is key for our effort to end our dependence on foreign oil and address the climate crisis while creating millions of new jobs that can't be outsourced" (U.S. Department of Energy, 2010b).

Approximately 73% of ethanol produced worldwide is fuel ethanol, 17% is beverage ethanol, and 10% is industrial ethanol (Sánchez and Cardona, 2008). Either as a fuel or as a gasoline enhancer, bioethanol is the most commonly used biofuel. As an oxygenator, it has higher oxygen content which reduces the amount of additives needed. Furthermore, in the presence of bioethanol, gasoline hydrocarbons are oxidized better which reduces the emission of CO and aromatic compounds. Bioethanol does not contaminate water sources, it is not toxic, and it has octane boosting properties (Thomas and Kwong, 2001).

Bioethanol can be produced from energy crops and lignocellulosic biomass. This process can be simple or complex depending upon the feedstock origin. Hence, the design and implementation of this process can involve just the fermentation of simple sugars or a multi-stage conversion of a complex matrix (such as lignocellulosic material) into bioethanol (Cardona and Sánchez, 2007). Fermentation is without a doubt one of the most important steps in the bioethanol process in which a microorganism (bacteria, yeasts, or filamentous fungi) metabolizes

the sugars present in the substrate to bioethanol (Figures 2 and 3). *S. cerevisiae* and *Z. mobilis* are the most common microorganisms used in this process (Gamage et al., 2010). However, fermentation with *Z. mobilis* could be inhibited by the high levels of salt and many industrial substrate sources have significant salt content (e.g. molasses), for instance, Bekers at al., (2000) reported the inhibition in the growth and ethanol production of *Z. mobilis* under salt concentrations of 0.6 M NaCl.

1. Saccharomyces cerevisiae

Saccharomyces cerevisiae, or "sugar fungus" from its Latin root, has been utilized by humans for thousands of years. It has been used to make dough rise and to produce ethanol for alcoholic beverages since it was discovered on the skins of grapes in the ancient years (British Broadcast Corporation, 2006). *S. cerevisiae*, also known as "brewer's yeast," is one of the most highly researched model organisms in the study of biology due to its well-known structure; it exists in single-cell form, or in pseudomycelial form; it reproduces by budding and it has the ability to ferment specific sugars which is a major factor that differentiates it from other yeasts (British Broadcast Corporation, 2006).

S. cerevisiae is classified in the fungi kingdom because it has a cell wall made of chitin, it has no peptidoglycan in its cell walls, and its lipids are ester-linked. It also uses a DNA template for protein synthesis and it has larger ribosomes than other microorganisms. However, it is classified as yeast, distinct from other fungi, because it is a unicellular organism that cannot form a fruiting body like other fungi. (Madigan and Martinko, 2006). *S. cerevisiae* is able to growth through both aerobic respiration and anaerobic fermentation, so it can survive in an oxygen-deficient environment for a considerable period of time, and it has both sexual and asexual

reproduction capabilities which allow the organism to live in many different environments (Madigan and Martinko, 2006).

S. cerevisiae produces 2 moles of adenosine triphosphate (ATP) per mole of glucose through the Embden-Meyerhoff-Parnas pathway (Figure 2.2) and uses only 6-carbon sugars such as glucose and fructose, as substrates in ethanol fermentation. *S. cerevisiae* is the most common microorganism used for the industrial production of bioethanol. It is principally used in the production of first generation bioethanol from sugar and starch feedstocks; It is a typical eukaryote with high ethanol fermentation yields and is relatively resistant to inhibitors—glucose (substrate) and ethanol (product) (Gamage et al., 2010). Nevertheless, *S. cerevisiae* is not resistant to certain concentrations of toxic compounds produced during acid hydrolysis so the lignocellulose hydrolyzate is commonly treated to remove toxic compounds before fermentation in order to maximize ethanol yields. Since the removal of toxic compounds can be expensive, many researchers have attempted to improve the resistance of *S. cerevisiae* during fermentation (Almeida, et al., 2009; Endo, et al., 2008; Larsson, et al., 2001).

There are an abundance of studies reporting successful ethanol production from by-products by *S. cerevisiae*. For instance, Romao et al. (2012) reported ethanol production from hydrolyzed soybean molasses with a fermentation time under optimum conditions of 14 h with acid hydrolysis increasing ethanol yields by 13.3% compared to nonhydrolyzed soybean molasses. The highest ethanol yield was 46% with sulfuric acid. Likewise, Letti et al. (2012) attained an ethanol yield of 89.3% of the theoretical maximum using *S. cerevisiae* LPB1 in soybean molasses. Siqueira et al. (2008) reported that soybean meal molasses is an important potential raw material for the production of bio-ethanol, offering a good balance of macro- and micro-nutrients for fermentation with supplementation or pH adjustment deemed unnecessary.

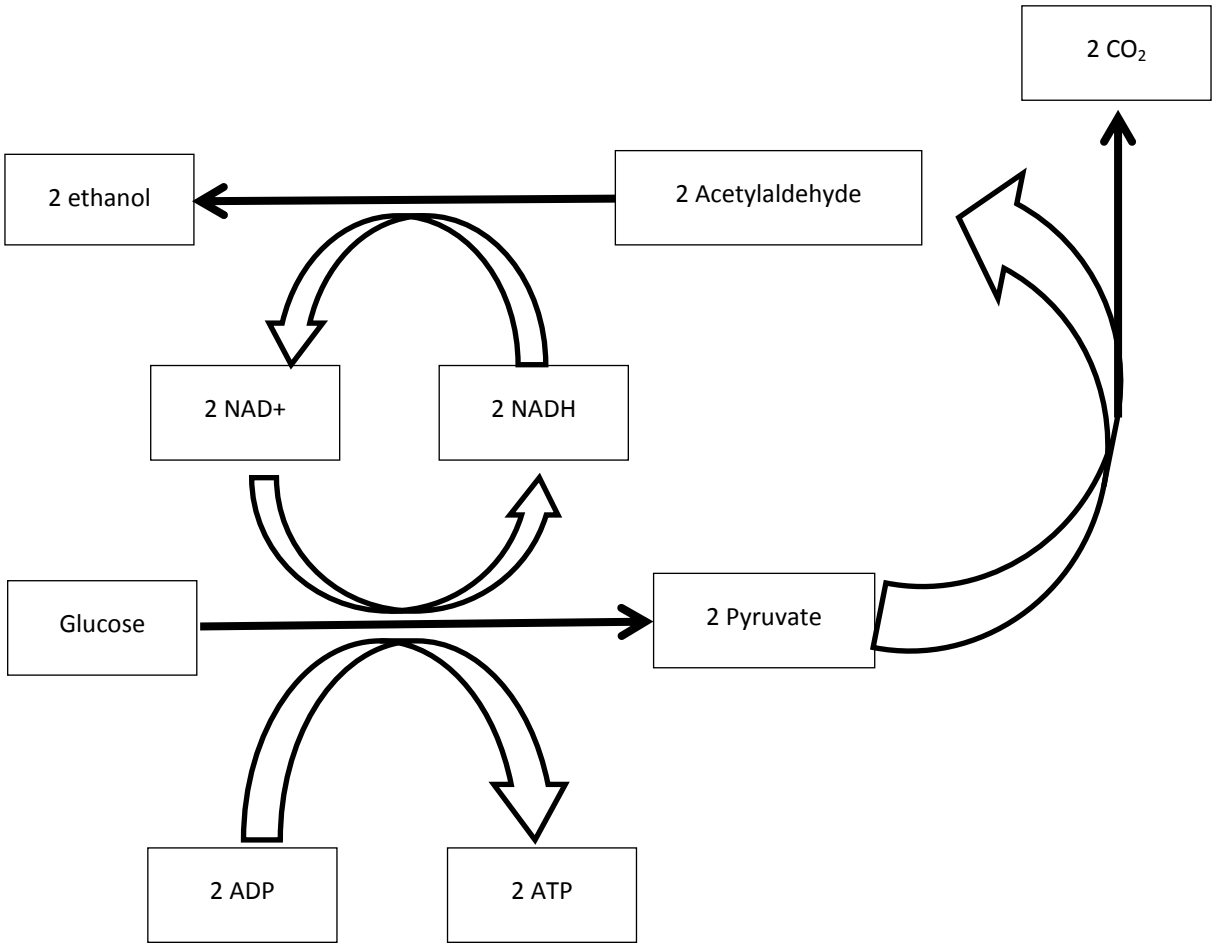


Figure 2.2. Anaerobic fermentation pathway (Muller, 2004).

Furthermore, the residue of bio-ethanol production (soybean vinasse) did not represent any environmental risk since it was used as a raw material for regeneration of energy. In this research, Siqueira et al (2008) obtained an ethanol productivity of 8.08 g/L, $Y_{P/S}$ 45.4% (g ethanol/ 100 g sugars), $Y_{X/S}$ 0.82% (g cells / 100 g sugars) and specific growth rate, μ_x 0.019 h^{-1}.

2. Zymomonas mobilis

Zymomonas mobilis was originally discovered in fermenting sugar-rich plant saps (e.g. in the traditional pulque drink (from agave sap) of Mexico, in palm wines of tropical Africa, and in ripening honey) (Swings and DeLey, 1977). It is an anaerobic, Gram-negative bacterium with the capacity to produce ethanol from glucose via the Entner-Doudoroff (2-keto-3-deoxy-6-phosphogluconate, KDPG) pathway in conjunction with the enzymes pyruvate decarboxylase and alcohol dehydrogenase.

Ethanol and carbon dioxide are the main products of catabolism when cells grow anaerobically on glucose (Sprenger G. 1996). *Z. mobilis* is considered one of the most promising bacteria for the industrial production of bioethanol from glucose. *Z. mobilis* is capable of a 97% theoretical ethanol yield with 5 times the volumetric productivity of *S. cerevisiae* (Balat, et al. 2008, Mohagheghi, et al., 2002). Additionally, the use of *Z. mobilis* is generally considered safe. Its extraordinary ethanol yield is due to the use of the Entner-Doudoroff pathway (Figure 2.3) instead of the Embden-Meyerhoff-Parnass pathway in anaerobic glucose metabolism. Thus, this bacterium produces less biomass and there is more carbon available for the production of ethanol (Dien et al., 2003). However, the wild strain will only utilize glucose, fructose, and sucrose as carbon sources in bioethanol fermentation (Gamage et al., 2010).

Letti et al. (2012) reported fermentation yields of *Z. mobilis* NRRL 806 in flasks and in a bench-scale batch reactor with 78.3% and 96.0% of the maximum theoretical yields with

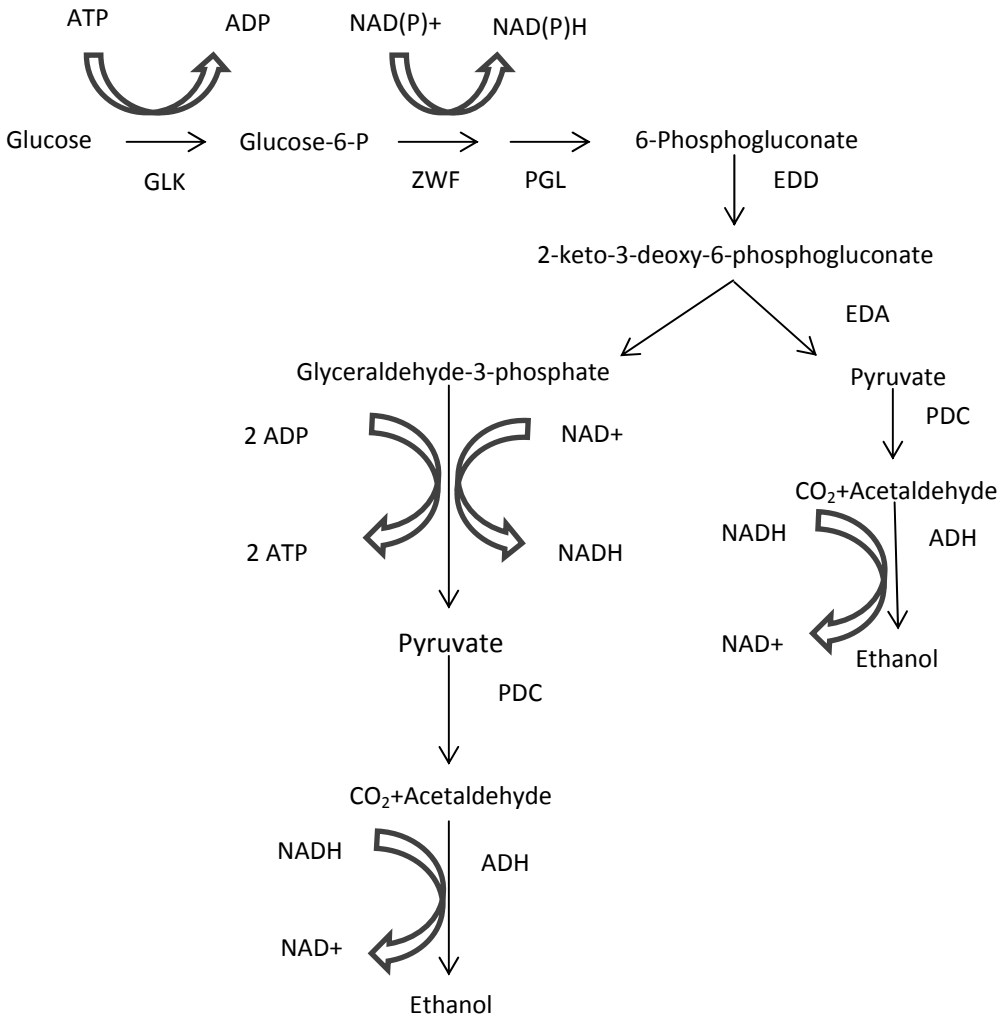


Figure 2.3. The Entner–Doudoroff pathway and *ethanologenesis for Z. mobilis.* The branch from glyceraldehyde-3-phosphate to pyruvate is identical to the Embden–Meyerhof–Parnas pathway. Abbreviations: GLK, glucokinase; ZWF, glucose-6-phosphate dehydrogenase; PGL, phosphogluconolactonase; EDD, 6-phosphogluconate dehydratase; EDA, 2-keto-3-deoxy-gluconate aldolase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase (Kalnenieks, 2006).

productions of 24.2 and 29.3 g/ L of ethanol, respectively. The medium consisted of dilute soybean molasses (150 g /L and 200 g/L of soluble solids) without additional nitrogen or salt supplementation. The microorganism was able to consume almost all the fructose and glucose content, but was not able to utilize galactose. Letti et al (2012) also compared the *Z. mobilis* and *S. cerevisiae* yields of ethanol production over total sugar and over sugar uptake, which were higher for the bacterium (0.25 and 0.49 g ethanol/g sugar, respectively) than for the yeast (0.24 and 0.46 g ethanol/ g sugar, respectively).

3. Growth kinetics and model development for the bioethanol fermentation of hydrolyzed SBM broths

The classical Monod model (Equation 1) is one of the most well-known kinetic models in fermentations, because it fits a wide range of biological data satisfactorily and is the most commonly applied unstructured- nonsegregated model of microbial growth (Shuler and Kargi, 2010; Doran, 1995); however, the classical semi-empirical Monod type equation cannot fit processes of fermentation well in many cases; for instance, it can describes substrate-limited growth only when growth is slow and population density is low, it has limited applicability at low substrate levels; that is why many modified types of Monod model have been made (Shuler and Kargi, 2010; Wang et al,, 2004; Doran, 1995). On the other hand, the logistic model (Eq. 2.2) , as a sigmoidal shaped model is the most popular one due to its "goodness of fit" and has been used frequently to describe the kinetic growth of microorganisms in different fermentation process (Sasikumar and Viruthagiri, 2008; Wang et al, 2004, Speers et al., 2003).

$$\mu = \frac{\mu_{max} S}{Ks+S} \qquad (2.1)$$

Where,

S is the substrate concentration (g/L)

μ is the specific growth rate (h^{-1})

μ_{max} is the maximum specific growth rate (h^{-1})

Ks is the saturation constant (g/L)

$$\frac{dx}{dt} = \mu_{max}\, x \left[1 - \frac{X}{Xm}\right] \quad (2.2)$$

Where,

dx/dt is the rate of biomass during the time of fermentation (g cell/ h)

μmax is the maximum specific growth rate (h^{-1})

X is the biomass concentration (g/L)

X_m is the maximum biomass concentration (g/L)

The logistic model has the following boundary conditions:

t=0, $X = X_0$, $S = S_0$, P = 0

By integration of equation 2.2, the kinetic model can be formulated. The biomass production rate yields the following equation 2.3 (the logistic equation for biomass production):

$$X = \frac{X_0\, X_{max}\, e^{\mu max t}}{X_{max} - X_0 + X_0 e^{\mu max t}} \quad (2.3)$$

CHAPTER III

Dilute Acid Hydrolysis of SBM at Atmospheric Pressure to Enhance Meal Protein Content and Produce Fermentable Sugars

A. Introduction

Soybean meal (SBM) is a co-product of soybean oil extraction generally used as animal feed (Kim et al., 2003). With a crude protein content ranging from 44 to 49% (Karr-Lilienthal et al., 2005b), SBM is a consistent and relatively inexpensive source of protein with high levels of essential amino acids (Dale et al., 2009; Smith, 2010; Da Silva et al., 2009). SBM contains significant levels of carbohydrates (35-42% d.b.), half of which are structural (cellulose, hemicelluloses, pectin, mannans, galactans, and xyloglucans), and the remaining, classified as non-structural, consist of approximately 17% d.b. low-molecular weight sugars (mono- and disaccharides), up to 8% d.b. oligosaccharides, and approximately 1% d.b. starch (Karr-Lilienthal et al., 2005b). Sucrose is the most abundant low-molecular weight sugar (6-8% d.b.) and the major oligosaccharides are stachyose (4-5%) and raffinose (1-2%) (Honig and Rackis, 1979).

When used as animal feed, of the soluble carbohydrate portion, only sucrose and starch are digested and absorbed by monogastric animals because they lack the enzyme α-galactosidase (Huisman et al., 1998). Although SBM contribute carbohydrates to the diet, its main function is to provide proteins (Waldroup, 2006). Therefore, a portion of the carbohydrates could be removed to create a protein-enriched meal that would have greater value, facilitate diet formulation, and potentially be used for new types of animal diets. A byproduct of the selective removal of carbohydrates would produce an important stream of fermentable sugars that could be used as substrates in the production of bioethanol, organic acids, and biomass. Considering that the production of SBM in the USA is projected to be 73.27 million metric tons at the end of

2012 (USDA, 2012), there is a significant potential for producing large quantities of soluble sugars via extraction for various purposes including bioethanol production.

Non-structural carbohydrates are the simplest to extract. From this group, glucose and sucrose are readily fermentable while oligosaccharides and starch need to be hydrolyzed to release glucose and fructose. Dilute acid hydrolysis is the most commonly used method of chemical hydrolysis. It is used as a pretreatment for enzymatic hydrolysis or to directly hydrolyze material to sugars (Qureshi and Manderson, 1995); however, it can produce toxic compounds, such as furfural and 5-hydroxymethylfurfural (5-HMF). 5-HMF is an aldehyde that is the product of the decomposition of fructose in acid conditions. These products are extremely undesirable in fermentation reactions because they inhibit glycolysis, particularly interfering with the activity of dehydrogenases, causing a reduction in growth rates and cell yields (Larsson et al., 2000; Lee and Lyer., 1999; Taherzadeh, 1999). Thus, the conditions of dilute acid hydrolysis must also be optimized to minimize the production of toxic compounds.

The objective of this chapter, which corresponds to Objective 1 of this dissertation, was to extract fermentable sugars from SBM with dilute sulfuric acid, which included the study of the effect of various time-sulfuric acid concentration combinations on the extent of sugar extraction, the quality of the remaining meal, and the production of 5-HMF.

B. Materials and Methods

1. Materials

SBM of an unidentified variety was obtained from a soybean processor in the state of Arkansas. In order to eliminate larger particles, the SBM was sieved using mesh with 2 mm-diameter orifices prior to hydrolysis. Reagents for analysis were sulfuric acid (96.5%) from J.T. Baker (Phillipsburg, NJ, USA), 5-HMF (99%) from Sigma Aldrich (St. Louis, MO, USA), and

sodium hydroxide (99%) from Sigma Aldrich. The standards for sugar analysis were glucose, arabinose, galactose, fructose, sucrose, stachyose, raffinose, and maltose; also obtained from Sigma-Aldrich.

2. Methods

Composition of the untreated soybean meal

Moisture content was determined by drying 10 g SBM in a conventional oven (VWR, Model 1310; Sheldon, Inc., Cornelius, OR, USA) at 115ºC until constant weight between readings (20-24 hours). The starch content was determined by the Enzyme Method 79-13 (A.A.C.C., 2000), the acid and neutral detergent-Fiber (AD-fiber, and ND-fiber) by an ANKOM-200 (Macedon, NY, USA), and the ash content by method A.O.A.C. 923.03 (A.O.A.C., 1990a). Crude protein content, sugar content, and color values were determined according to the methods described in the following sections.

Dilute acid hydrolysis

The dilute acid hydrolysis of SBM was conducted by treating soybean meal samples with low concentrations of sulfuric acid (0.5, 0.72, 1.25, 1.78, and 2.0% w/v) for 1.0, 3.2, 8.5, 13.8, and 16.0 hours at a ratio of 1:5 (10 grams of fresh SBM in 50 mL H_2SO_4 solution). The hydrolysis was conducted in 125 mL Erlenmeyer flasks with screw caps in a water bath (VWR Model 1227; Sheldon, Inc., Cornelius, OR, USA) at 80 ºC with horizontal shaking of 150 RPM. Levels of sulfuric acid concentration and hydrolysis time were arranged according to a central composite rotatable experimental design (CCRD) (Table 3.1). Once individual hydrolysis times were attained, the flasks were immersed in an ice water bath until the samples reached room temperature. The reaction was stopped by adding NaOH pellets to raise the pH to a value between 5 and 5.5. Samples were subsequently centrifuged at 4500 RPM for 35 minutes at 10ºC.

The supernatant was isolated, filtered through Whatman #4 filter paper (110 mm Ø; Whatman Plc., Kent, UK), and stored at -20ºC for analysis of fermentable sugars, total sugars, 5-HMF, and furfural. The pellet was also stored at -20ºC for analysis of crude protein content and color.

Table 3.1. Central Composite Rotatable Design for the hydrolysis of soybean meal with H_2SO_4 at 80°C

Std. Order	**Run Order**	**Codified Levels**		**Real Levels**	
		Time (X1)	Concentration (X2)	Time (h)	Concentration (%)
1	1	-1	-1	3.2	0.72
11	2	0	0	8.5	1.25
5	3	-1.4142	0	1.0	1.25
3	4	-1	1	3.2	1.78
6	5	+1.4142	0	16.0	1.25
10	6	0	0	8.5	1.25
8	7	0	+1.4142	8.5	2.00
13	8	0	0	8.5	1.25
12	9	0	0	8.5	1.25
9	10	0	0	8.5	1.25
7	11	0	-1.4142	8.5	0.50
4	12	1	1	13.8	1.78
2	13	1	-1	13.8	0.72

Fermentable and total sugars analysis

Fermentable sugars in the liquid fraction were determined by High-Performance Size Exclusion Chromatography with Refractive Index (HPSEC-RI) detection according to Giannoccaro et al. (2008). The equipment was a Waters HPSEC-RI (Milford, MA, USA), consisting of a 515 HPLC pump with an injector valve with a 50-µL sample loop, and a 2410 refractive index detector maintained at 40 ºC. Sugars were separated by two Shodex columns (Showa Denko America, Inc., New York, NY, USA)—an OH Pack SB-804 HQ (300 x 8 mm) followed by an OH Pack SB-802 HQ (300 x 8 mm)—maintained at 55 ºC by a column heater. These were preceded by a Shodex OH pack SB-G (50 x 6 mm) guard column. The mobile phase was 0.1M $NaNO_3$ with 0.2% NaN_3 (8.499 g $NaNO_3$ + 0.2 g NaN_3 in 1 L distilled water) run at an isocratic flow rate of 0.4 mL/min for 46 minutes. The sugars—Arabinose (Arab), Galactose (Gal), Glucose (Glc), Fructose (Fruc), Sucrose (Suc), Raffinose (Raf), Stachyose (Stac), Maltohexaose (Malthex), and Maltotetraose (Maltetra)—were quantified using a six-point standard calibration curve. Total sugar content in the liquid fraction was determined by the phenol sulfuric acid method (Dubois, 1956), following a sugar extraction with water and using glucose as the standard. (Giannoccaro et al., 2008).

5-Hydroxymethylfurfural (5-HMF) analysis

The concentration of 5-HMF in each sample (liquid fraction) was determined with a Prominence Ultra Fast Liquid Chromatography (UFLC) (Shimadzu, Kyoto, Japan) equipped with a Shimadzu C-18 column (50 mm x 4.6 mm), a SPD-20 AV UV-visible detector, a DGU-$20A_3$ degasser, and LC-20AB pumps. The mobile phase was acetonitrile: water (30:70 v/v) (Ko et al., 2008) maintained at a flow rate of 0.5mL/min. The run time was five minutes with an

injection volume of 500 uL. Prior to the analysis, samples were adequately diluted in distilled water and then filtered through a 0.45 μm nylon membrane syringe filter.

Crude protein content

The crude protein content was determined by the nitrogen combustion method (A.O.A.C., 1990b) using an Elementar Variomax Instrument (Elementar Americas, Inc. Mt. Laurel, NJ, USA). The SBM solid fraction was dried at 60ºC for 24 h prior to analysis and a 220 mg sample of the dried SBM solid was used for analysis.

Color

The color of the untreated SBM and the color of the solid fractions isolated after hydrolysis were determined by a Minolta Chroma Meter (CR-300, Minolta Camera Co. Ltd, Osaka, Japan) according to the methods described by Humphries et al. (2004) and Fratianni et al. (2005). The Chroma Meter measures the color co-ordinates within the Commission Internationale l'Eclairage (CIE) Lab three-dimensional color space (L*a*b*). Each CIE L*, a*, b* value was the average of three readings. The Chroma Meter was calibrated with a white tile and a black card initially and then periodically throughout analysis.

Experimental design and statistical analysis

The effect of acid concentration and time on the fermentable sugar extraction yields and the other variables (total sugar, crude protein, 5-HMF, and color) were studied using a central composite rotatable design (CCRD) with 2 factors, 5 levels, and 13 runs (Table 3.1). The responses were fitted to a quadratic equation (equation 3.1). The response surface design was analyzed with Minitab 15.1.30.0 (State College, PA, USA) using full quadratic models with and without interaction terms to describe the response surface.

$$Y= \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (3.1)$$

C. Results and Discussion

1. Chemical composition of the untreated soybean meal

The starting material, untreated SBM, contained 48.72 ± 0.01% d.b. crude protein, 22.15 ± 0.07% d.b. total soluble sugars, 2.80 ± 0.04 % d.b. starch, 4.32 ± 0.01% d.b. acid detergent fiber, 15.64%± 0.01% d.b. neutral detergent fiber—that includes the insoluble cell wall components, cellulose, hemicellulose and lignin (A.O.A.C., 1990c, Van soest et al., 1991)—5.87 ± 0.02% d.b. ash, and 7.16 ± 0.08% moisture. The untreated SBM contained 7.91 ± 0.21% d.b. of fermentable sugars—primarily sucrose and small amounts of glucose and fructose. The SBM composition was within the range reported by others authors (Dale et al., 2009, Da Silva et al., 2009, Grieshop et al., 2003).

2. Fermentable sugars and total soluble sugars in the SBM

The coefficients for the quadratic equation that represents the responses of fermentable sugars and total soluble sugars extracted from SBM with dilute acid are shown in Table 3.2 with a detailed list of individual soluble sugars in Table 3. Total sugar content fit a quadratic model that contained all the coefficients (Table 3.2) except the interaction between time and concentration ($\beta_1\beta_2$) The maximum yield of fermentable sugars was 21.0 g/100g SBM d.b. that was obtained following treatments with 1.9-2% H_2SO_4 for 6-16 h. Glucose and fructose are the sugars with highest concentration that were likely generated as a result of the breakdown of sucrose, stachyose, and raffinose (Table 3.3), which was also suggested by other authors (Montilla et al., 2009, Iloukhani et al., 2001). The lowest yield of fermentable sugars, 7.6 g/100g SBM d.b., was obtained when treated with 0.5-0.7% H_2SO_4 for 1-5 h. A comparison of the initial fermentable sugars contained in the untreated meal (7.91 g/100g) against the maximum yield obtained after acid treatment (21 g/100g) shows the effectiveness of the treatment to hydrolyze

some components, presumably oligosaccharides, and release fermentable sugars. Stachyose and raffinose concentrations were low in comparison with the other sugars after treatments (Table 3.3). Likely, this was the result of complete or partial hydrolysis of these carbohydrates into glucose, fructose and/or fructose.

The maximum concentration of total soluble sugars in the liquid fraction was 22.1 g/100 g SBM d.b. following treatment with 1-1.75% H_2SO_4 for 4-13 h (Figure 3.1) while the minimum was 6.4% d.b. following treatment with 0-0.2% H_2SO_4 for 0-3 h. The highest content of total soluble sugars attained in this work is comparable to the total sugars content in SBM molasses (17-21%) reported by Oliveira et al. (2005) using a mixture of ethanol-water for the extraction.

Table 3.2. Coefficients for the full quadratic model for the treatment of soybean meal with dilute H_2SO_4 at 80°C

Response	β_o	β_1	β_2	β_{12}	B_{11}	B_{22}	Mean Square	Model P-value	Model F-value	Lack-of-fit (P-value)
Fermentable sugars	14.788 (<0.000)	1.693 (<0.045)	3.967 (<0.000)	–	–	–	4.358	<0.001	17.08	< 0.983
Total soluble sugars	23.334 (<0.000)	0.222 (<0.732)	0.696 (<0.30)	–	- 1.756 (<0.031)	-2.372 (<0.008)	3.1478	<0.01	8.57	< 0.828
5-HMF	0.042 (<0.000)	0.031 (<0.000)	0.055 (<0.000)	0.034 (<0.000)	–	0.0199 (<0.000)	0.00003	<0.01	89.82	< 0.034
Crude protein	55.005 (<0.000)	-1.414 (<0.010)	-2.030 (<0.001)	-2.355 (<0.004)	–	-1.017 (<0.052)	1.412	<0.052	5.18	< 0.112
Color (L-value)	44.137 (<0.000)	-4.399 (<0.000)	-4.318 (<0.000)	–	–	–	2.365	< 0.082	3.83	< 0.077
Color (a-value)	8.864 (<0.000)	–	-0.519 (<0.023)	–	–	–	0.309	< 0.023	6.96	< 0.359
Color (b-value)	24.177 (<0.000)	-2.127 (<0.001)	-1.470 (<0.010)	–	–	–	1.748	<0.001	15.29	< 0.306

Values in parenthesis represent the P-value for each coefficient

Table 3.3. Sugar yields estimated by HPSEC-RI following acid treatment of SBM. Values are reported as g sugar/100 g dry SBM±SE

		Sugars						
Time (h)	**H_2SO_4 (%w/v)**	**Maltohexaose**	**Stachyose**	**Raffinose**	**Sucrose**	**Glucose**	**Fructose**	**Fermentable Sugars***
3.2	0.72	1.42±0.01	0.08±0.03	0.20±0.06	0.19±0.02	1.65±0.00	5.60±0.18	8.86±0.42
8.5	1.25	2.84±0.01	bdl	0.05±0.17	2.82±0.01	5.06±0.08	9.37±0.01	20.1±0.10
1.0	1.25	1.22±0.02	bdl	0.14±0.04	0.68±0.03	2.70±0.04	6.81±0.12	11.4±0.34
3.2	1.78	2.16±0.30	bdl	0.09±0.03	1.33±0.21	4.09±0.23	8.83±0.61	16.4±0.90
16.0	1.25	3.21±0.12	bdl	0.10±0.02	1.19±0.14	3.45±0.28	8.70±0.13	16.5±0.49
8.5	1.25	1.84±0.01	bdl	0.12±0.08	0.52±0.01	3.02±0.01	6.92±0.01	12.3±0.08
8.5	2.00	3.96±0.01	bdl	bdl	1.74±0.01	5.56±0.08	9.73±0.01	21.0±0.12
8.5	1.25	2.59±0.01	bdl	0.14±0.08	1.71±0.01	3.62±0.09	8.83±0.01	16.7±0.13
8.5	1.25	2.31±0.01	bdl	0.11±0.09	0.98±0.01	3.90±0.07	7.75±0.01	14.9±0.12
8.5	1.25	2.04±0.01	bdl	0.10±0.04	0.66±0.00	3.53±0.08	7.51±0.01	13.7±0.13
8.5	0.50	0.00±0.00	0.23±0.07	0.06±0.02	1.40±0.09	1.90±0.25	5.32±0.35	8.62±0.65
13.8	1.78	4.11±0.34	bdl	bdl	1.30±0.14	5.07±0.07	8.64±0.14	19.1±0.53
13.8	0.72	2.10±0.01	bdl	0.17±0.01	0.60±0.01	2.87±0.11	6.88±0.01	12.4±0.13
Untreated SBM		bdl	5.56±0.01	1.92±0.01	7.25±0.17	0.61±0.06	0.05±0.01	7.91±0.21

*Fermentable sugars=Maltohexaose + Sucrose + Glucose + Fructose
bld: below detection limit

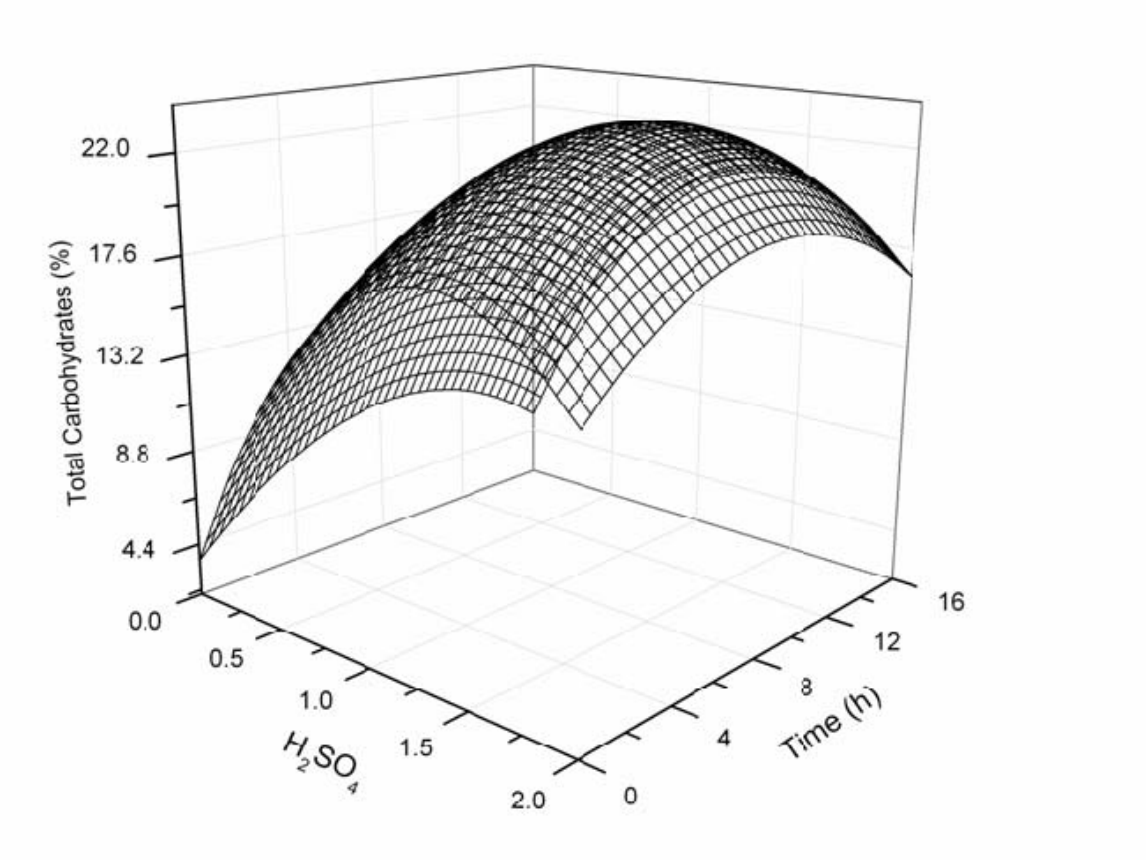


Figure 3.1. Total carbohydrates (soluble sugars) content in the liquid fraction of dilute-acid-treated soybean meal determined by the phenol sulfuric acid method

3. 5-Hydroxymethylfurfural (5-HMF)

The concentration of 5-HMF followed the quadratic model with the exception of the second order effect of time (Table 3.2). Production of 5-HMF increased with increasing concentrations of acid and time (Figure 3.2). The maximum level of 5-HMF was approximately 0.21 g/L for the longest treatments (above 12 h) and the highest concentration of acid (2 % H_2SO_4). Previous research showed that ethanol production with *Z. mobilis* is lowered by 20 to 40% when the 5-HMF concentration in the fermentation broth is above 0.09 g/L (Pienkos and Zhang, 2009). *S. cerevisiae*, on the other hand, is less susceptible to 5-HMF. To reduce yeast ethanol production by 50%, the concentration of 5-HMF in the fermentation broth has to be around 8 g/L (Clark and Mackie, 1984). Thus, the amount of 5-HMF generated with the conditions used in this study would not considerably affect the ethanol yield using *S. cerevisiae*, but likely would affect ethanol production when using *Z. mobilis*. To utilize *Z. mobilis*, the level of 5-HMF during dilute acid treatment would need to be reduced. One method may be the implementation of the two-stage dilute-acid process, as recommended by Taherzadeh and Karimi (2007), since fewer fermentation-inhibiting compounds are formed during two-stage hydrolysis. Another method would be the application of activated carbon after the dilute acid treatment as reported by other authors (Mussatto and Roberto, 2004; Lee et al., 1999).

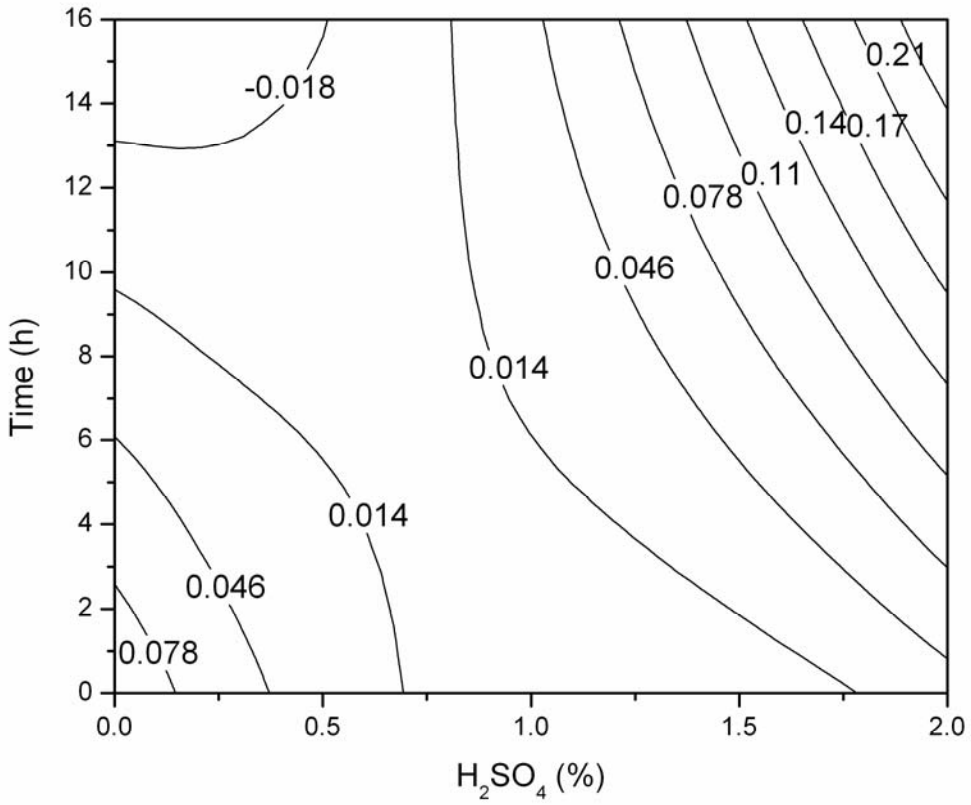


Figure 3.2. Concentration of 5-Hydroxymethylfurfural (5-HMF) in the liquid fraction of acid-hydrolyzed soybean meal

4. Crude Protein

For protein content, all terms of the quadratic model (Table 3.1) except the quadratic term for time were significant (Table 3.2). The maximum protein concentration (58% d.b.) in the solid fraction was obtained when SBM was treated with 1.2-1.7% H_2SO_4 for 1-2.6 h at 80ºC, while the minimum (48% d.b.) was observed when SBM was treated with 1.9-2.0% H_2SO_4 for 13-16 h (Figure 3.3). These results show that as the acid concentration increased, the protein concentration decreased. Overall, there was a remarkable improvement in the protein concentration from 48% (untreated SBM) to 58% with the shorter treatment times and lower acid concentrations. In contrast, with the higher acid concentrations and longer times, the protein concentration was significantly reduced. This could be caused by Maillard reactions (or non-enzymatic browning) which is a common occurrence when amino groups of proteins and reducing sugars are exposed to high temperatures (Richardson, 2001). The maximum protein content attained in this research is similar to that obtained by others—SBM with 58% protein using ethanol-water to extract sugars (Oliveira et al., 2005) and distillers grain with 58-61% when treated with dilute acid (Tucker et al., 2004).

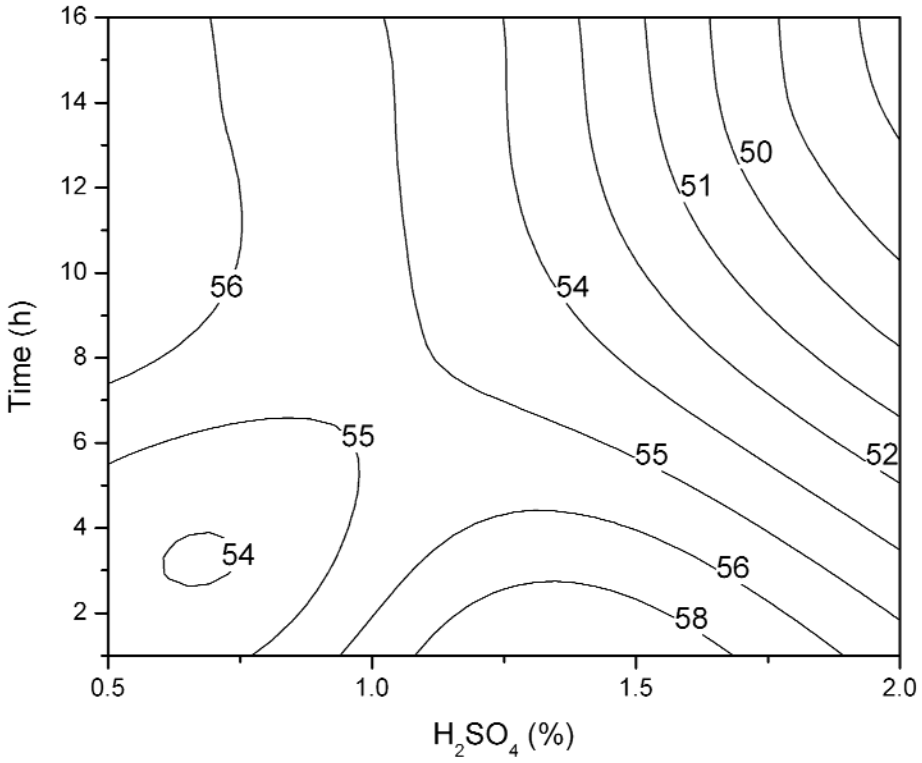


Figure 3.3. Crude protein content of the solid fraction of acid-hydrolyzed soybean meal

5. Color

Color values—L* and b*—followed a linear response with time and concentration (Table 3.2). Whereas value a* only follow a linear response with concentration. The maximum CIE L* value (62.00) was attained when SBM was treated with 0-0.25% H_2SO_4 for 0-1.8 h, which was the closest value to the untreated sample (62.31) (Table 3.4). In contrast, when SBM was treated with 1.7-2.0% H_2SO_4 for 10-16 h, the minimum L* value was 37.00. Therefore, as the acid concentration and treatment time increased, the dark color of the SBM hydrolyzate increased. However, the maximum CIE a* value of the solid fraction was 9.9 when SBM was treated with 0.2% H_2SO_4 (for any length of time) and the lowest value was 8.2 when treated with 1.9% H_2SO_4 (for any length of time). All the values were relatively close to that of the original SBM (8.72), which could indicate the scale from green to red was not considerably affected by the concentration of acid or the length of treatment time. Finally, the CIE b* value was clearly affected by high acid concentrations and long treatment times as evidenced by the low value obtained (20.00) compared to the untreated sample (27.12) when the SBM was hydrolyzed with 1.6-2.0% H_2SO_4 for 13-16 h. The maximum CIE b* was 30.00 when the SBM was treated with 0-0.25% H_2SO_4 for 0-2 h. The color values are fairly comparable to those obtained by Oliveira (2005) with reported maximum L*, a*, and b* values of 70.0, 4.0 and 20.0, respectively, and minimum L*, a*, and b* values of 65.0, 2.0, and 17.0, respectively. The differences in color between the two studies are likely due to the differences in the extraction, since Oliveira et al. (2005) utilized ethanol and water which were less severe treatments over the SBM color.

Table 3.4. Color values means (L*a*b*) of the solid fraction of acid-hydrolyzed soybean meal ± SE

Time (h)	H_2SO_4 (%w/v)	**L***	**a***	**b***
3.2	0.72	54.0±1.2	10.3±0.2	30.2±0.3
8.5	1.25	42.7±2.1	8.2±0.4	22.3±1.3
1.0	1.25	52.4±1.8	8.2±0.4	26.9±1.1
3.2	1.78	45.4±1.4	8.3±0.1	25.1±0.4
16.0	1.25	41.4±0.6	8.4±0.1	22.4±0.6
8.5	1.25	43.4±1.3	8.7±0.2	23.5±1.1
8.5	2.00	40.7±0.6	8.1±0.4	21.9±0.4
8.5	1.25	43.6±1.6	8.8±0.4	23.1±1.4
8.5	1.25	44.4±0.7	9.6±0.2	25.3±0.7
8.5	1.25	44.9±0.4	8.4±0.1	23.1±0.4
8.5	0.50	50.2±0.8	9.5±0.1	25.9±0.1
13.8	1.78	33.7±0.6	9.1±0.1	21.9±0.1
13.8	0.72	46.1±0.8	9.3±0.1	22.8±0.4
SBM (dry)		65.4±0.1	8.7±0.2	37.6±0.3
SBM (wet)		63.3±1.5	5.6±0.6	27.1±0.1

D. Conclusions

Dilute acid hydrolysis of SBM with 1.9 to 2% H_2SO_4 for 7 to 16 h resulted in as much as a 21% d.b. of fermentable sugars with low concentration of stachyose and raffinose with relatively low 5-HMF levels (less than 0.21 g/L); thus, acid-hydrolyzed SBM could be a suitable and promising source of sugars for bioethanol production as well as other important products in the food industry such as lactic or acetic acid, xylitol, and microbial biomass. Furthermore, the protein content of the solid fraction, following extraction, increased from 48 to 58% when SBM was treated with 1.25- to 1.7% H_2SO_4 for 0.5 to 2.5 h without considerably altering the original SBM color; thus, this by-product could be a good source of protein in animal feed. Overall, a good balance of dilute acid hydrolysis to produce fermentable sugars from SBM while enhancing its protein content ranged from treatments for 2.5-7.0 h with 1.7-2% H_2SO_4.

CHAPTER IV

Dilute Acid Hydrolysis of SBM at High Temperatures

A. Introduction

Dilute acid hydrolysis of SBM with 1.9 to 2% H_2SO_4 for 7 to 16 h at 80^{o}C resulted in as much as a 21% d.b. of fermentable sugars (Chapter 3). These hydrolyzates had less than 0.21 g/L of 5-HMF which make them suitable substrates for ethanolic or other fermentations. Nevertheless, improvement in the fermentable sugars is possible by increasing the temperature and pressure of the SBM dilute acid hydrolysis. There is evidence that higher temperatures and pressures allow more than 80% of the hemicellulose to be hydrolyzed as well as some fractions of cellulose converted to glucose (Buhner and Agblevor, 2004; Larsson et al., 2000; Lee et al., 1999). Sulfuric acid hydrolysis at high temperatures and pressures can further improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to high reaction rates and more complete cellulose hydrolysis (Esteghlalian et al., 1997, McMillan, 1994, Morjanoff and Gray, 1987). Furthermore, hydrolysis at higher temperatures and pressures could reduce the reaction times and production of toxic substances during the process (Taherzadeh and Karimi, 2007). However, one of the important issues is the degradation of sugars and the formation of further undesirable by-products, such as furfural, 5-HMF, acetic acid, levulinic acid, uronic acid, 4-hydroxybenzoic acid, vanillic acid, vanillin, phenol, cinnamaldehyde, and formaldehyde that reduce sugar yields and inhibit ethanol production during fermentation (Buhner and Agblevor, 2004; Larsson et al., 2000; Lee et al., 1999).

High temperatures and pressures during acid hydrolysis of the hemicellulose fraction of lignocellulosic material also prepares the cellulose fraction for further conversion by enzymes

and presents promising opportunities to reduce the cost of this kind of bioprocess (Jacobsen and Wyman, 2000). Therefore, dilute acid hydrolysis of SBM at high temperatures and pressures may improve the SBM functionality by increasing the concentration of fermentable sugars which may augment the profitability of bioethanol or any other metabolite production where fermentable sugars are the main substrate. Additionally, the increase in protein concentration of the remaining solid after acid hydrolysis may improve its value-added and commercial uses as an animal feed.

The aim of this research, which corresponds to Objective 2 of this dissertation, is to determine the optimal conditions of dilute acid hydrolysis under high temperatures of SBM to produce fermentable sugars while enhancing its protein content. Also, the effects of the treatments on the concentration of toxic compounds [5-hydroxymethylfurfural (5-HMF) and furfural] and the SBM color were evaluated.

B. Materials and Methods

1. Materials

Soybean meal (SBM) of an unidentified variety was obtained from a soybean processor in Arkansas. The SBM was sieved using a mesh with 2 mm-diameter orifices prior to the hydrolysis process. The main reagents used for analysis were sulfuric acid (96.5%) from J.T. Baker (Phillipsburg, NJ, USA), 5-HMF (99%) from Sigma Aldrich (St. Louis, MO, USA), furfural (98%) from TCI America, and sodium hydroxide (99%) from Sigma Aldrich. The standards for sugar analysis (glucose, arabinose, galactose, fructose, sucrose, stachyose, raffinose, maltoses) were also obtained from Sigma-Aldrich.

2. Methods

Acid hydrolysis at high temperatures

The hydrolysis, at temperatures between 105 and 135^{o}C, was conducted in duplicate in a Tuttnauer 2340E Steam Autoclave (Tuttnauer USA, Delran, NJ). Samples were treated at 3 temperatures, 4 concentrations of sulfuric acid, and 3 time durations arranged according to a split-plot experimental design with the concentration of H_2SO_4 as the split-plot factor and temperatures and times as the whole plot (Table 4.1). Hydrolyses were carried out in 125 mL Erlenmeyer flasks with screw cap at a ratio of 1:5 (10 g SBM: 50 mL H_2SO_4 solution). The reactions were stopped by adding NaOH pellets to raise the pH to a value between 5 and 5.5. Then samples were centrifuged at 3900 x g for 35 minutes at 10ºC. The supernatant was filtered through Whatman #4 filter paper (Whatman Plc., Kent, UK). The supernatant was isolated and stored at -20ºC for analysis of fermentable sugars, total sugars, 5-HMF, and furfural. The pellet was also stored at -20ºC for analysis of crude protein content and color.

Table 4.1. Experimental design for the hydrolysis of soybean meal with H_2SO_4 at high temperature

Day	**1**						**2**						**3**					
Run	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Temp.	T1	T1	T1	T2	T1	T2	T1	T3	T2	T3	T2	T1	T1	T1	T1	T2	T1	T3
Conc.	C3	C0	C0	C3	C2	C2	C3	C0	C0	C3	C2	C2	C1	C1	C2	C3	C0	C2
Time	t2	t1	t2	t2	t1	t2	t3	t3	t2	t2	t1	t2	t1	t3	t3	t3	t3	t1

Day	**4**						**5**						**6**					
Run	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Temp.	T1	T1	T1	T3	T3	T2	T1	T3	T2	T1	T1	T2	T3	T1	T1	T3	T1	T2
Conc.	C1	C3	C1	C1	C3	C3	C0	C2	C1	C0	C0	C1	C1	C1	C2	C0	C2	C2
Time	t2	t1	t1	t2	t3	t1	t2	t2	t2	t1	t3	t3	t1	t2	t2	t3	t3	t3

Day	**7**						**8**						**9**					
Run	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
Temp.	T3	T2	T3	T1	T3	T3	T2	T2	T3	T2	T2	T1	T2	T1	T3	T2	T3	T2
Conc.	C3	C0	C2	C3	C0	C3	C0	C1	C0	C3	C0	C3	C2	C3	C2	C3	C2	C1
Time	t1	t3	t1	t3	t1	t2	t1	t2	t2	t2	t2	t1	t2	t2	t3	t3	t3	t1

Day	**10**						**11**						**12**					
Run	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
Temp.	T2	T3	T3	T1	T3	T2	T3	T3	T2	T2	T3	T1	T3	T2	T2	T3	T2	T3
Conc.	C0	C1	C3	C2	C1	C3	C0	C3	C1	C0	C0	C1	C1	C2	C1	C2	C2	C1
Time	t1	t2	t1	t1	t3	t1	t2	t3	t3	t3	t1	t3	t3	t3	t1	t2	t1	t1

T1= 105°C (5PSI), T2= 120°C (15PSI), T3= 135°C (32PSI), C0=0%, C1=0.5%, C2=1.25%, C3=2%, t1= 15 min., t2= 30 min., and t3= 45 min.

Analytical methods

The composition of the soybean meal, the fermentable sugars, 5-HMF and furfural content of the liquid fraction, and the crude protein content and color of the solid fraction were determined using the methods described in Chapter 3. Acetic acid concentration in the liquid hydrolyzate was analyzed using the method described by McGinley and Mott (2008) with a Waters HPSEC-RI (Milford, MA, USA) system consisting of a 515 HPLC pump with an injector valve with a 50 μL sample loop and a 2410 refractive index detector maintained at 40 ºC. Acetic acid was separated in a column Rezex ROA-organic acid H+ (8%) (150 x 7.80 mm) with a guard column KJ0-4282 (Phenomenex, Torrance, CA) maintained in a column heater at 60 ºC. The mobile phase was 0.005N H_2SO_4 at a flow rate of 0.6 mL/min; the run time was 15 minutes.

Cellulose degradation in the hydrolyzed SBM

To determine surface degradation of SBM particles after the hydrolysis treatments, images of selected samples were taken with a scanning electron microscope (SEM FEI ESEM XL-30. Philips, USA.). Samples were gold-coated with a sputter coater (EMITECH, SC7620, Quorum Technologies Ltd, UK.) prior to imaging.

Additionally, cellulose degradation was evaluated using a method described by Updegraff (1969). Following the removal of lignin, hemicellulose and xylosan materials with acid/nitric acid reagent, the cellulose was hydrolyzed with 67% sulfuric acid. The cellulose concentration was determined by the anthrone method using a cellulose calibration curve.

Statistical Analysis

Analysis of variance and the mean yield of fermentable sugars at the end of the hydrolysis, by Fisher's least significant difference procedure ($\alpha = 0.05$), were carried out with SAS, Version 9.2, software (SAS Institute Inc., Cary, NC, USA).

Cellulose degradation data were analyzed with JMP® Version 9.0.0 (SAS institute Inc., Cary, NC, USA). Analysis of variance and Tukey-Kramer test ($\alpha = 0.05$) were also carried out to analyze differences in the mean cellulose composition at following acid hydrolysis.

C. Results and Discussion

1. Fermentable sugars

Most of the interactions among factors (temperature, H_2SO_4 concentration, and time), for each dilute acid treatment concentration, had significant differences ($p<0.05$) in the means of fermentable sugar yields in the liquid fraction of hydrolyzed SBM. Sulfuric acid concentration was the main factor affecting the fermentable sugars yield. The two highest concentrations of H_2SO_4 (1.5% and 2%) led to the highest fermentable sugars yields (Figure 4.1a).

The initial concentration of fermentable sugars in the untreated SBM, extracted with water (Chapter 3), was 7.9% d.b. (dry basis) (Figure 4.1a). After dilute acid hydrolysis at 135°C for 45 min with 2% H_2SO_4 (T3C3t3), the concentration of fermentable sugars in the SBM liquid fraction was 32.2% d.b. which is 24.3% d.b. greater than the untreated SBM. The lowest fermentable sugar concentration (8.3% d.b.) in the liquid fraction was obtained with one of the mildest treatment conditions, 105°C, 0.5% H_2SO_4, and 15 min (T1C1t1). Thus, dilute acid hydrolysis under high temperatures and short times is effective at extracting a considerable amount of fermentable sugars.

Of the fermentable sugars, the most abundant in the majority of treatments were glucose and fructose (Table 4.2a,b,c) and were likely generated as a result of the breakdown of sucrose, raffinose, and stachyose, as well as, to a small extent, the hydrolysis of the larger polysaccharides, cellulose and hemicellulose (Iloukhani et al., 2001; Montilla et al., 2009). Clearly, for instance, after acid hydrolysis, the sequential bonds in the stachyose structure

(gal(α1→6)gal(α1→6)glc(α1↔2β)fru) were mostly hydrolyzed to generate glucose and fructose. Galactose was not detected by the HPLC analysis, so it probably was destroyed by most acid treatments.

The maximum glucose concentration (19.2% d.b.) was obtained by treatment at 135°C with 2% H_2SO_4 for 45 min (T3C3t3) (Table 4.2c), whereas the maximum fructose concentration(8.2% d.b.) was reached by treatments at 105°C, with 2% H_2SO_4, and for 30 min (T1C3t2) (Table 4.2a) and also 120°C, 2% H_2SO_4, and 15 min (T2C3t1) (Table 4.2b). Sucrose was present in low concentrations after most of the treatments except for the lowest acid concentrations where the concentration was similar to the untreated SBM (Table 4.2a). Overall, sucrose concentration was reduced as H_2SO_4 concentration increased regardless of the treatment time or temperature; in contrast, the concentration of maltohexaose increased with increasing H_2SO_4 regardless of treatment time or temperature (Table 4.2). The increase in maltohexaose was likely the result of the breakdown of the larger polysaccharides, cellulose and hemicellulose which were not hydrolyzed significantly under lower severity treatments. Stachyose and raffinose were completely hydrolyze under all the temperatures combined with 1.25% and 2% of H_2SO_4 and mostly hydrolyzed by 0.5% of H_2SO_4.

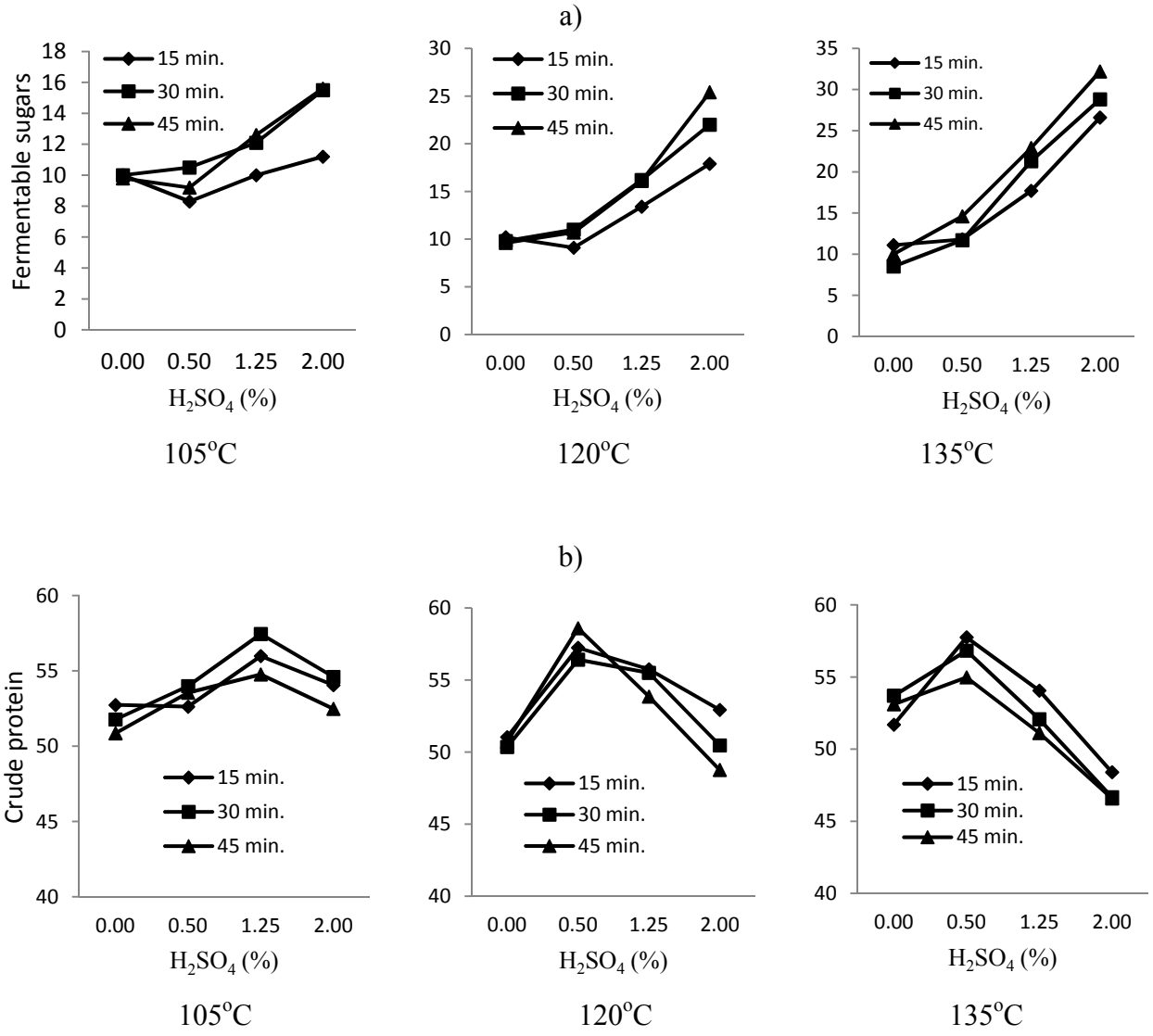


Figure 4.1. a) Mean fermentable sugars (in d.b.). b) Crude protein content (in d.b.) of the soybean meal (SBM) hydrolyzed with H_2SO_4 at high temperatures. For untreated SBM, protein was 48.1% d.b.

LSD to compare fermentable sugar means: for values at different temp*time combinations is 1.53%; for values at different concentrations (with same temp*time combination) is 1.45%. LSD to compare crude protein means: for values at different temp*time combinations is 1.89%, for values at different concentrations (with same temp*time combination) is 1.82%

Table 4.2a. Means of Sugar yields after acid treatment of soybean meal at 105°C (T1). Values are reported as g sugar/100 g dry SBM ± SE.

Sugar	Untreated	Time (min)	H_2SO_4 (%)			
			0%	0.5%	1.25%	2%
Maltohexaose	-	15	bdl	bdl	1.0 ± 0.05	1.1 ± 0.04
		30	bdl	bdl	1.5 ± 0.23	1.7 ± 0.23
		45	bdl	bdl	1.6 ± 0.13	1.8 ± 0.03
Stachyose	5.6 ± 0.03	15	5.5 ± 0.21	5.7 ± 0.08	bdl	bdl
		30	5.4 ± 0.23	5.2 ± 0.49	bdl	bdl
		45	5.1 ± 0.14	bdl	bdl	bdl
Raffinose	1.9 ± 0.04	15	1.1 ± 0.33	1.5 ± 0.11	bdl	bdl
		30	2.4 ± 0.11	bdl	bdl	bdl
		45	2.2 ± 0.02	bdl	bdl	bdl
Sucrose	7.3 ± 0.02	15	8.1 ± 0.01	6.4 ± 0.08	0.6 ± 0.06	0.4 ± 0.03
		30	7.9 ± 0.02	4.9 ± 0.48	0.6 ± 0.08	0.6 ± 0.11
		45	7.7 ± 0.15	3.3 ± 0.17	0.5 ± 0.11	0.5 ± 0.16
Glucose	0.62 ± 0.04	15	1.4 ± 0.13	1.4 ± 0.04	3.1 ± 0.25	3.4 ± 0.25
		30	1.6 ± 0.02	2.6 ± 0.46	3.7 ± 0.12	5.0 ± 0.37
		45	1.6 ± 0.12	2.2 ± 0.13	3.8 ± 0.04	5.5 ± 0.35
Fructose	0.05 ± 0.01	15	0.5 ± 0.01	0.5 ± 0.01	5.3 ± 0.28	6.5 ± 0.22
		30	0.5 ± 0.06	3.0 ± 0.01	6.3 ± 0.35	8.2 ± 0.09
		45	0.5 ± 0.01	3.7 ± 0.35	6.7 ± 0.26	7.8 ± 0.19

bdl: below detection limit

Table 4.2b. Means of sugar yields after acid treatment of soybean meal at 120°C (T2). Values are reported as g sugar/100 g dry SBM ± SE

Sugar	Untreated	Time (min)	H_2SO_4 (%)			
			0%	0.5%	1.25%	2%
Maltohexaose	-	15	bdl	bdl	1.5 ± 0.18	2.0 ± 0.22
		30	bdl	1.2 ± 0.04	2.2 ± 0.22	3.2 ± 0.06
		45	bdl	1.6 ± 0.14	2.7 ± 0.12	4.3 ± 0.06
Stachyose	5.6 ± 0.03	15	5.0 ± 0.33	bdl	bdl	bdl
		30	5.1± 0.13	bdl	bdl	bdl
		45	4.6 ± 0.18	bdl	bdl	bdl
Raffinose	1.9 ± 0.04	15	2.5 ± 0.23	bdl	bdl	bdl
		30	2.3 ± 0.11	bdl	bdl	bdl
		45	2.6 ± 0.71	bdl	bdl	bdl
Sucrose	7.3 ± 0.02	15	7.9 ± 0.45	2.9 ± 0.48	0.6 ± 0.11	0.5 ± 0.02
		30	7.8 ± 0.09	1.3 ± 0.3	0.6 ± 0.01	0.5 ± 0.07
		45	7.7 ± 0.04	0.5 ± 0.01	0.4 ± 0.01	0.7 ± 0.06
Glucose	0.62 ± 0.04	15	1.7 ± 0.49	2.2 ± 0.16	4.2 ± 0.04	7.2 ± 0.29
		30	1.4 ± 0.06	3.2 ± 0.21	5.6 ± 0.15	10.7± 0.62
		45	1.1 ± 0.04	3.2 ± 0.25	5.4 ± 0.03	13.0± 0.16
Fructose	0.05 ± 0.01	15	0.7 ± 0.01	4.0 ± 0.30	7.1 ± 0.18	8.2 ± 0.21
		30	0.6 ± 0.01	5.4 ± 0.32	7.7 ± 0.42	7.6 ± 0.23
		45	0.8 ± 0.01	5.4 ± 0.04	7.6 ± 0.06	7.3 ± 0.01

bdl: below detection limit

Table 4.2c. Means of sugar yields after acid treatment of SBM at 135°C (T3). Values are reported as g sugar/100 g dry SBM ± SE

Sugar	Untreated	Time (min)	H_2SO_4 (%)			
			0%	0.5%	1.25%	2%
Maltohexaose	-	15	bdl	1.6 ± 0.04	3.6 ± 0.47	4.7 ± 0.04
		30	bdl	2.6 ± 0.45	5.5 ± 0.11	6.2 ± 0.32
		45	bdl	3.6 ± 0.37	6.3 ± 0.40	8.0 ± 0.23
Stachyose	5.6 ± 0.03	15	4.5 ± 0.01	bdl	bdl	bdl
		30	4.2 ± 0.23	bdl	bdl	bdl
		45	3.7 ± 0.54	bdl	bdl	bdl
Raffinose	1.9 ± 0.04	15	3.1 ± 0.28	bdl	bdl	bdl
		30	2.7 ± 0.03	bdl	bdl	bdl
		45	3.1 ± 0.10	bdl	bdl	bdl
Sucrose	7.3 ± 0.02	15	8.6 ± 0.14	0.8 ± 0.03	0.4 ± 0.01	bdl
		30	6.9 ± 0.16	0.4 ± 0.06	0.1± 0.01	bdl
		45	7.5 ± 0.37	0.7 ± 0.09	bdl	bdl
Glucose	0.62 ± 0.04	15	1.7 ± 0.23	3.4 ± 0.12	6.1 ± 0.22	14.7 ± 0.10
		30	1.0 ± 0.14	3.1 ± 0.05	8.8 ± 0.35	17.3 ± 0.33
		45	1.7 ± 0.44	3.8 ± 0.21	9.8 ± 0.36	19.2± 0.10
Fructose	0.05 ± 0.01	15	0.9 ± 0.16	6.0 ± 0.13	7.6 ± 0.01	7.2 ± 0.21
		30	0.6 ± 0.08	5.7 ± 0.15	6.9 ± 0.16	5.2 ± 0.06
		45	0.7 ± 0.02	6.5 ± 0.30	6.7 ± 0.17	5.0 ± 0.37

bdl: below detection limit

2. Crude protein

The lowest concentrations of H_2SO_4 did not appreciably reduce the protein content in the solid fraction of SBM during the treatments nor did the treatments extract significant amounts of fermentable sugars in the liquid fraction (Figures 4.1a and 4.1b). The largest increase in crude protein yields were reached by treatments with 0.5% H_2SO_4 (Figure 4.1b). After the dilute acid treatments of SBM, the greatest concentration of crude proteins (58.6% d.b.) was obtained by treatment at 120°C with 0.5% H_2SO_4 for 45 min (T2C1t3), whereas the lowest (46.58% d.b.) was obtained by treatment at 135°C with 2% H_2SO_4 for 45 min (T3C3t3). Thus, as the acid concentration increased, the protein concentration in the SBM decreased (Figure 4.1b). These results demonstrate that the protein structures are highly affected by acid concentration. This was likely the result of Maillard reactions (or non-enzymatic browning), which is a common occurrence when amino groups of proteins and reducing sugars are exposed to high temperatures for long periods of time (Richardson, 2001). This may also be confounded by the protein denaturation at high temperatures and longer times.

In summary, there was a remarkable improvement in the protein concentration from 48.1% d.b. (untreated SBM) to 58.6% d.b. with one of the less concentrated treatments—treatment at 120°C with 0.5% H_2SO_4 for 45 min (T2C1t3)—which is comparable to the protein content obtained by Oliveira et al., (2005) when they used ethanol-water to extract sugars from SBM. Similarly, 58-61% d.b. protein content was reported by Tucker et al. (2004) following treatment of distillers grain with dilute acid. With higher acid concentrations and longer treatment times, however, the protein concentration was not significantly greater which may be attributed to Maillard reactions.

3. 5-Hydroxymethylfurfural (5-HMF), furfural, and acetic acid

Production of 5-HMFand furfural increased as concentrations of acid, time and temperature increased (Figure 4.2a and 4.2b). The maximum levels of 5-HMF and furfural were 0.002 g/L and 0.32 g/L, respectively, with treatment 135°C, 2% H_2SO_4, and 45 min. (T3C3t3). The maximum 5-HMF concentration is lower than the concentration (0.7 g/L) reported by Panagiotopoulos et al. (2012) after dilute acid treatments in barley straw; however, Saha et al (2005a) did not detect any 5-HMF or furfural when they hydrolyzed rice hull with dilute H_2SO_4 (1% v/v) at 120-190ºC. Likewise, Saha et al (2005b) did not find measurable amounts of 5-HMF in wheat straw hydrolyzed with dilute H_2SO_4 (0.5% v/v) at 180 ºC for 15 minutes, but they observed furfural (32 mg/g wheat straw d.b.) and acetic acid (24 g/g wheat straw d.b.).

Ethanol production by *Z. mobilis* is lowered by 20-40% when the 5-HMF concentration in the fermentation broth is above 0.09 g/L (Pienkos and Zhang, 2009). *S. cerevisiae*, on the other hand, is less susceptible to 5-HMF; for instance, to reduce the production of ethanol by 50%, the concentration of 5-HMF in the fermentation broth has to be around 8 g/L (Clark and Mackie, 1984). Thus, the amount of 5-HMF generated with the conditions used in this study would not considerably affect the ethanol yield in a fermentation process using *S. cerevisiae* or *Z. mobilis*.

However, Szengyel and Zacchi (2000) reported that the activity of the enzymes cellulase and ß-glucosidase is affected by the concentration of furfural. With an increase in furfural concentration from 0 to 1.2 g/L, cellulase activity decreased from 1.32 to 0.73 FPU (filter paper unit)/mL and the ß-glucosidase activity decreased by 50%. Hence, it is possible that the furfural concentration accumulated after some of the treatments, especially the high acid concentrations and temperatures, could affect further enzymatic treatments applied to the hydrolyzed SBM.

Therefore, it would be desirable to reduce the level of furfural during the dilute acid treatment. One way is by using the two-stage dilute-acid process recommended by Taherzadeh and Karimi (2007) since fewer fermentation-inhibiting components are formed during two-stage hydrolysis. The other alternative to reduce the furfural formed during the acid hydrolysis is by applying activated carbon after the treatment (Mussatto and Roberto, 2004; Lee et al., 1999).

The highest acetic acid concentration (Figure 4.2c) was generated by the most severe treatments—135°C, 2% H_2SO_4, and 45 min. (T3C3t3) and 135°C, 2% H_2SO_4, and 30 min (T3C3t3) (0.87 and 0.85 g/L, respectively)—and no acetic acid was observed in the mildest treatments. Acetic acid is mainly formed from acetylated sugars derived from hemicellulose (Taherzadeh and Karimi, 2007; Larsson et al., 2000). Thus, the acetic acid concentration is augmented by increasing severity of hydrolysis conditions since the same trend was noted for fermentable sugars and hemicellulose/cellulose degradation.

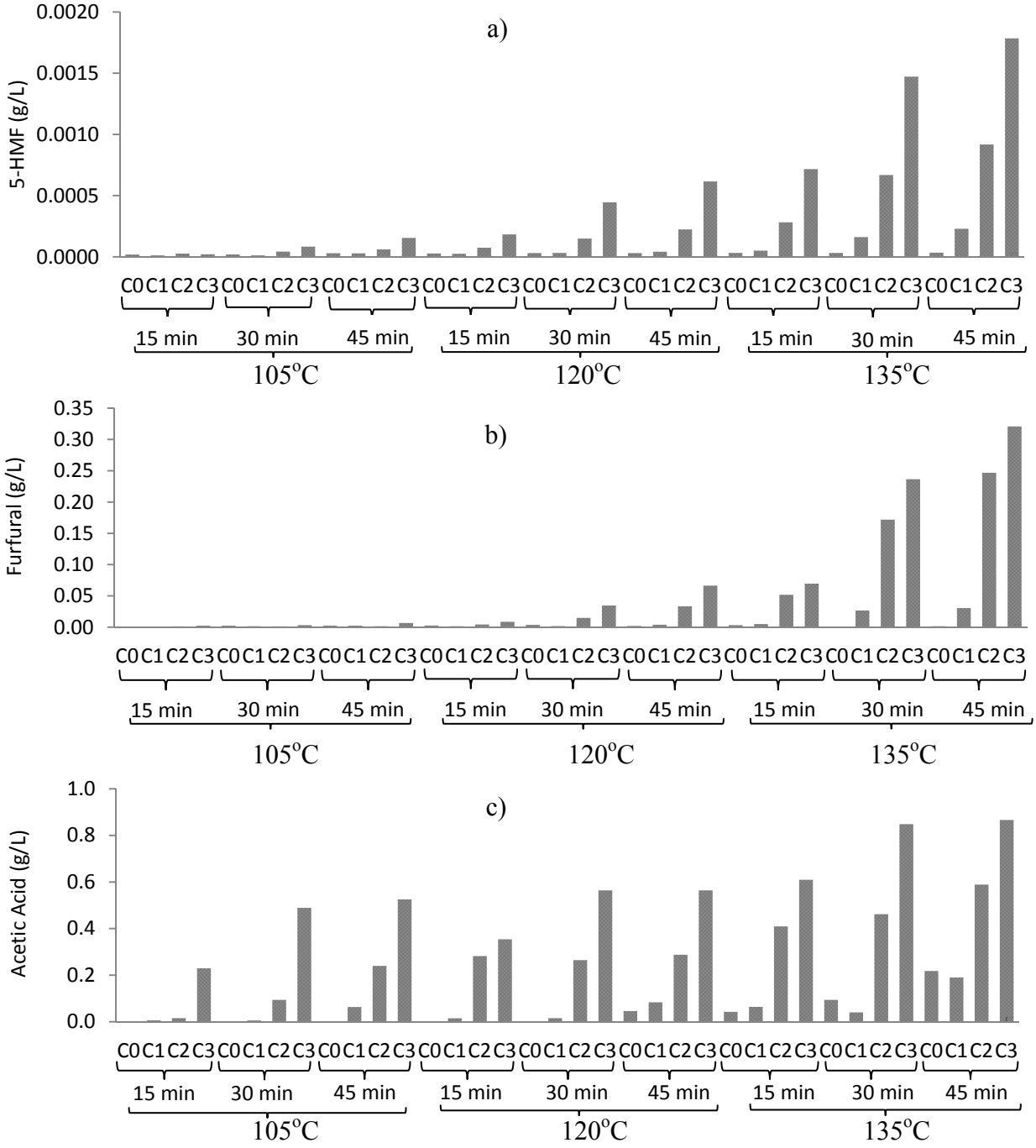


Figure 4.2. a) 5-HMF, b) Furfural, and c) Acetic acid means for SBM hydrolyzed with H_2SO_4 at the following concentrations: C0=0%, C1=0.5%, C2=1.25%, C3=2%. For 5-HMF, LSD to compare fermentable sugars means at different Temp*times combinations is 4.3 10^{-5} and 4.1 10^{-5} for different concentrations and the same Temp*time combinations. For Furfural, LSD to compare compare fermentable sugars means at different Temp*times combinations is 5.9 $10^{-3}$and 5.7 10^{-3} for different concentrations at the same Temp*time combinations. For acetic acid, LSD to compare fermentable sugars means at different Temp*times combinations is 0.036 and 0.026 for different concentrations at the same Temp*time combination.

4. Color

In the solid fraction of SBM, the maximum L* value (68.3) was obtained when SBM was treated at 105°C with 0% H_2SO_4 for 15 min (T1C0t1) and also was the closest value to the untreated sample (69.6) (Figure 4.3a). In contrast, the lowest L* value was 33.8 when SBM was treated at 135°C with 2% H_2SO_4 for 45 min (T3C3t3). Overall, L* decreased when samples were subjected to high temperatures (Farroni and Buera, 2012). Therefore, as the acid concentration, temperature, and time increased, the dark color of the treated SBM also increased. This may be attributed to the Maillard reactions—also referred to as nonenzymatic browning or glycation—produced under these conditions (Maillard, 1912; Hodge, 1953) .

The maximum a* value was 9.0 when SBM was treated at 135°C with 1.25% H_2SO_4 for 45 min (T3C2t3) while the lowest value was 4.5 when treated at 105°C with 0.5% H_2SO_4 for 15 min (T1C1t1), which was close to that of the untreated SBM (4.6). Thus, the scale from green to red was not considerably affected by less rigorous treatments but was affected by the more severe treatment combinations (Figure 4.3b).

The b* value was, overall, not highly affected by most treatments as evidenced by the lowest value obtained (19.6) compared to the original sample (28.8) under the most severe conditions (135°C, 2% H_2SO_4, 45 min or T3C3t3). The maximum b* values were 32.1 and 31.2 with treatments at 105°C for 30 min with 1.25% (T1C2t2) and 2% H_2SO_4 (T2C1t1), respectively (Figure 4.3c). Overall, a* decreased when samples were treated at high temperatures. The color values are fairly comparable to those obtained by Oliveira et al. (2005) with reported maximum L*, a*, and b* values of 70.0, 4.0 and 20.0, respectively, and minimum L*, a*, and b* values of 65.0, 2.0, and 17.0, respectively. Besides differences in raw materials, other differences in color

between the two studies are likely due to the variations in the extraction since Oliveira et al. (2005) utilized a mix of ethanol and water for the hydrolysis.

5. Cellulose degradation in the hydrolyzed SBM

Cellulose composition in the hydrolyzed SBM was not highly affected by the majority of the high temperature and pressure treatments. This is likely due to the recalcitrance of cellulose to dilute acid hydrolysis with maximum glucose yields only attainable with temperatures greater than 220ºC; however, at temperatures less than 200ºC, most of the hemicellulose (more than 80%) can be hydrolyzed by dilute acid hydrolysis (Larsson et al. 2000, Taherzadeh, 1999; Lee and Lyer, 1999).

Treatment means at 105°C were not significantly different from the untreated SBM (Figure 4.4) with a cellulose concentration of 7.53% d.b. Hence, the low temperature treatment did not affect the original SBM concentration of cellulose. However, the cellulose content of samples treated at higher temperatures were significantly different from the untreated SBM ($p<0.0001$, $\alpha=0.05$, STDError=0.0728). The degradation of cellulose increased as the temperature, acid concentration and reaction time increased. The maximum degradation of cellulose (50%) was the result of the most rigorous treatment combination—135°C, 2% H_2SO_4, 45 min (T3C3t3). Thus, the native cellulose structure was partially disintegrated by the most intense treatments—high temperatures, high acid concentration, and longer times—and the resulting material could be more susceptible to hydrolysis with enzymes, such as cellulase and β-glucosidase, that break down cellulose to glucose.

For additional evidence of the treatment effects on the structural carbohydrates, SEM images (Figure 4.5) showed that SBM particles are degraded after acid hydrolysis in distinct levels depending upon the treatment strength. The surface particles are clearly different between

the treatments. Surface particles from the untreated SBM (Figure 5a) have smooth and clean outer layers whereas surface particles from the highest treatments (Figures 4.5b, 4.5c) are dispersed with micro particles and irregular surfaces. This may be evidence that cellulose fibers are agglomerates of individual cellulose micro-fibers as was reported by Corredor (2008) in soybean hulls. The images demonstrate that there are reductions in the external surface area of cellulose which potentially decreases the mass transfer resistance in the molecule; thus the cellulosic material may be more accessible during enzymatic treatment (Zhang and Lynd, 2004).

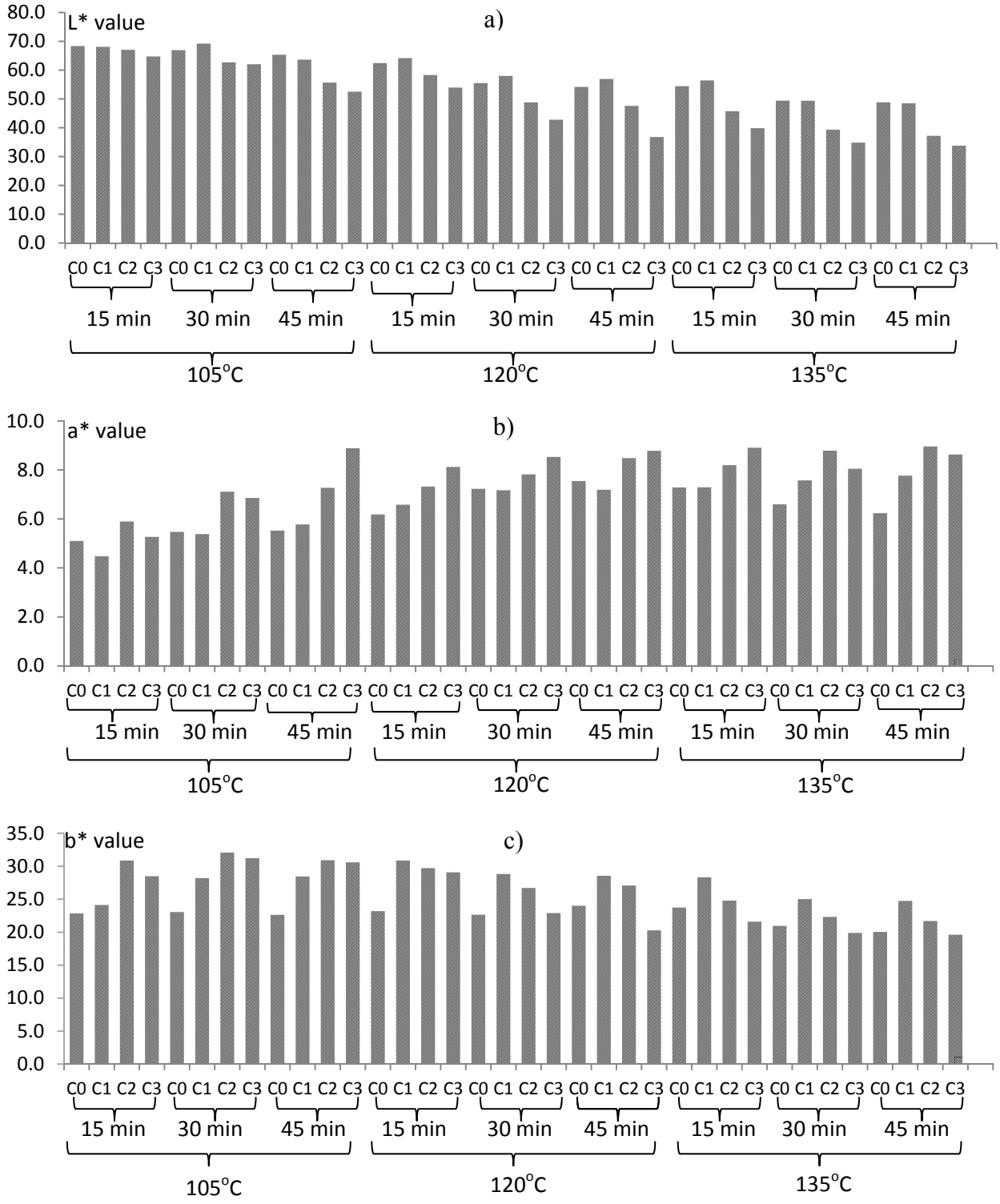


Figure 4.3. Color value means for soybean meal hydrolyzed with H_2SO_4 at high temperature. a) L*values (0=black, 100= white); b) a*values (-a*= green, +a*= red); c) b*values (-b*= blue, +b*= yellow). C0=0%, C1=0.5%, C2=1.25%, C3=2%. Untreated SBM: L = 69.6, a= 4.6, b=28.8

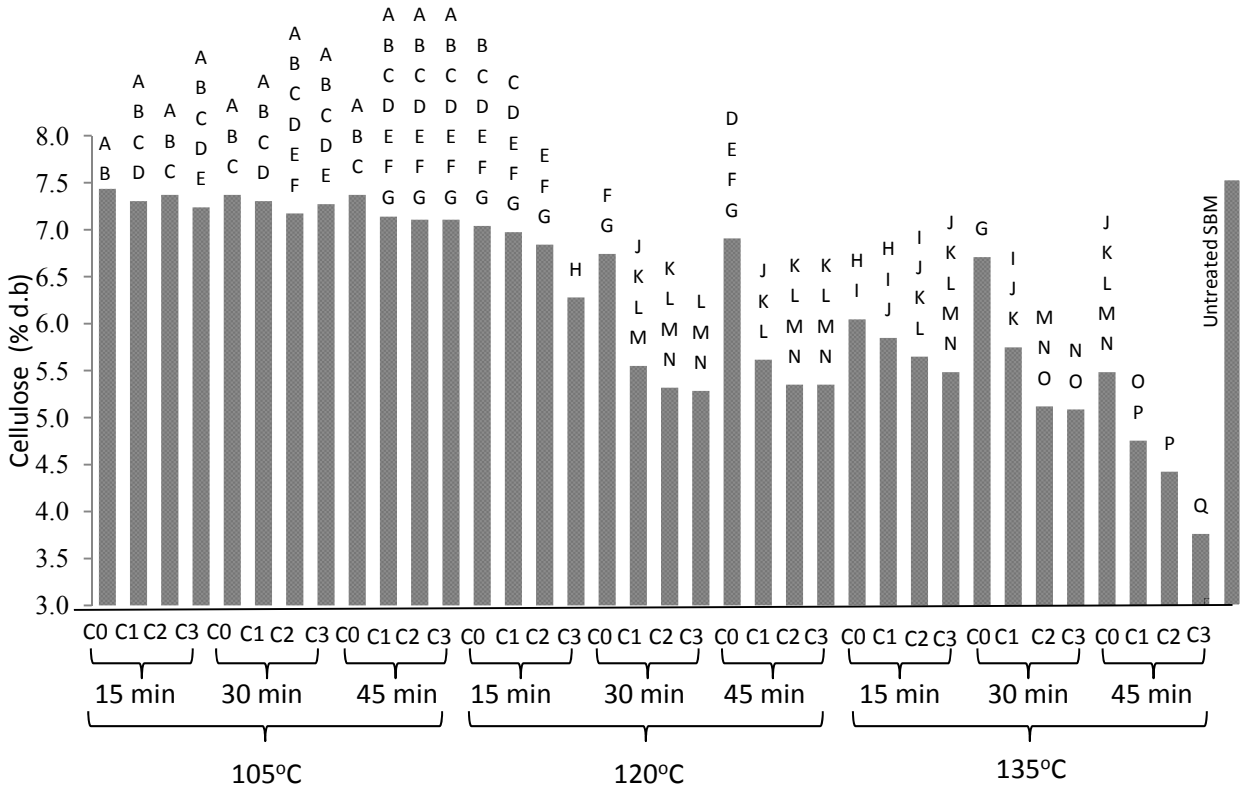
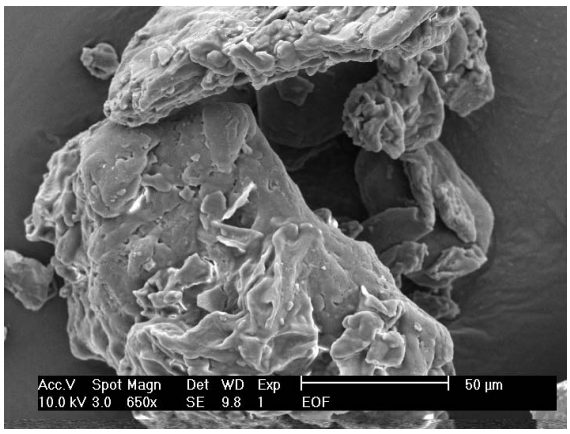
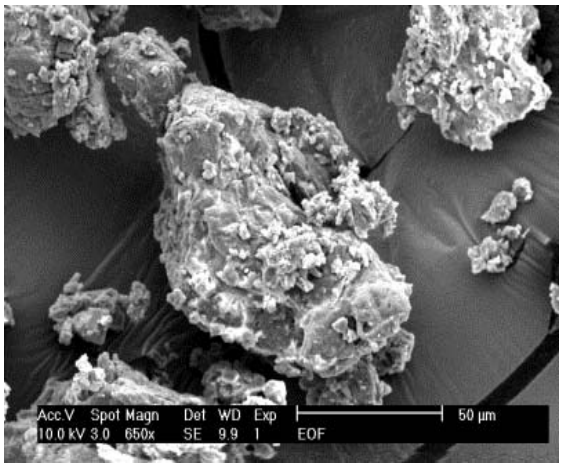


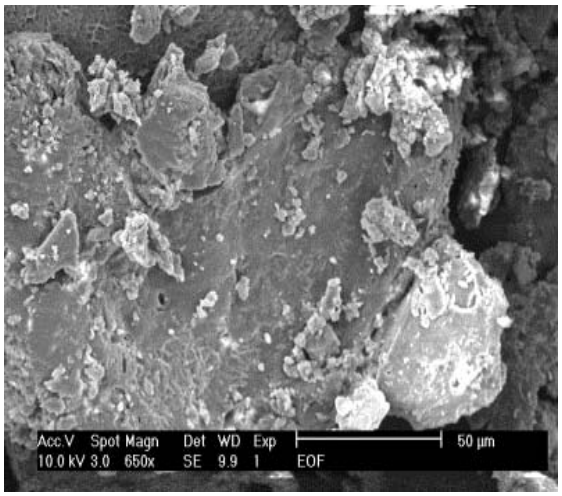
Figure 4.4. Cellulose concentration (% d.b.) in SBM after hydrolysis with dilute acid. C0=0%, C1=0.5%, C2=1.25%, C3=2% H_2SO_4. Untreated SBM: 7.54 % d.b. Treatments with same letter are not significantly different ($p<0.0001$, $\alpha=0.05$, SE=0.072).



a)



b)



c)

Figure 4.5. Scanning electron microscope images of soy bean meal treated with dilute acid at high temperatures. a) Original SBM (No treatment), b) SBM after treatment at 135°C with 0.5% H_2SO_4 for 45 min, c) SBM after treatment at 135°C with 2% H_2SO_4 for 45 min.

D. Conclusions

Dilute acid hydrolysis of SBM with the highest temperature (135°C), highest acid concentration (2% H_2SO_4), and longest treatment time (45 min) generated 32.2% d.b. fermentable sugars with relatively low 5-HMFand furfural levels (0.0018 g/L and 0.32 g/L, respectively) indicating that dilute acid-hydrolyzed SBM could be a suitable and promising source of fermentable sugars for the bioprocess industry. Furthermore, the degradation of cellulose increased as the temperature, acid concentration and reaction time increased with the maximum cellulolytic degradation (50%) produced under the same intense treatment conditions—135°C, 2% H_2SO_4, 45 min (T3C3t3). Following partial disintegration of the cellulose structure, it is also possible that the remaining material could be more susceptible to hydrolysis with enzymes. In contrast, the protein content of the solid fraction was not improved by the intense treatments. Only one of the less rigorous treatments (120°C, 0.5% H_2SO_4, and 45 min) increased the protein content (from 48.1% d.b. to 58.6% d.b) without considerably altering the SBM original color; thus, this by-product could be a better source of protein substitute in animal feed than the original SBM. In conclusion, treatment at 120°C with 1.5% H_2SO_4 for 30 min (T2C2t2) had the best balance between high concentrations of fermentable sugars (21.3% d.b.) in the liquid fraction and crude protein (52.1% d.b.) in the solid fraction without a detrimental change in the original color of the SBM solid fraction.

CHAPTER V

Enzymatic Treatment of Soybean Meal Hydrolyzates and Detoxification with Activated Carbon

A. Introduction

In Chapter 4, soybean meal was hydrolyzed with dilute acid at temperatures above 100^{o}C for various durations and with different concentrations of acid. The end products of these treatments were liquid and solid fractions rich in fermentable sugars and protein, respectively. It was also shown that the solid fraction underwent important lignocellulosic structure modifications, which would permit better performance of further enzymatic treatments to further improve the fermentable sugars yield.

Before the enzymatic hydrolysis of lignocellulosic materials, an acid pretreatment step is generally used to modify the structure of the matrix to generate a substrate highly susceptible to enzymes (Kumar and Wyman, 2009; Vlasenko et al.,1997). After the acid pretreatment, the amorphous cellulose structure is more accessible to enzymatic action (e.g. cellulose) due to the decreased mass transfer resistance (Zhang and Lynd, 2004). However, the efficacy of cellulase depends on the presence of inhibitors—such as 5-HMF, furfural and acetic acid— produced during the acid hydrolysis; therefore, a detoxification step is needed to reduce the concentration of these inhibitors (Szengyel and Zacchi, 2000). Activated carbon is commonly used to remove most of these inhibitors and its efficiency depends on the pH, contact time, temperature, and concentration (Mussatto and Roberto, 2004).

In a matrix like SBM, the presence of lignin and hemicellulose make the enzymatic hydrolysis more complicated than that of pure cellulose. Lignin reduces the amount of cellulose available because it acts as a competitive cellulose adsorbent (Eriksson et al., 2002; Bernardez et al., 1993; Ooshima et al., 1990; Sutcliffe and Saddler, 1986). Hence, lignin and hemicellulose

removal and/or redistribution have an important effect on observed rates of enzymatic hydrolysis (Cherboglazov et al., 1988; Converse, 1993; Lynd et al., 2002).

Cellulases, including β-glucosidases, are very specific enzymes that hydrolyze cellulose into glucose by breaking the 1,4-beta-D-glycosidic linkages in cellulose (Béguin and Aubert, 1994). The *Trichoderma reesei* cellulase mixture contains numerous catalytically active proteins with at least two cellobiohydrolases (CBH1 and CBH2), five endoglucanases (EG1–5), β-glucosidases, and hemicellulases, which have been identified by 2D electrophoresis (Vinzant et al., 2001). CBH1, CBH2, and EG2 are the three main components of the *T. reesei* cellulase cocktail, representing 60 ± 5%, 20 ± 6%, and 12 ± 3% of total cellulase protein, respectively (Nidetzky and Claeyssens, 1994; Goyal et al., 1991; Kyriacou et al., 1987; Knowles et al., 1987). The action of CBH1 and CBH2 result in a gradual decrease in the degree of polymerization (DP) of cellulose (Kleman-Leyer et al.,1992, 1996; Srisodsuk et al., 1998).

The aim of this research was to evaluate the enzymatic hydrolysis of the SBM pretreated with H_2SO_4 as indicated in Chapter 4. The experiment included samples that yielded the highest fermentable sugar concentrations of the SBM pretreated with acid, which were subjected to treatments with cellulase and ß-glucosidase with and without detoxification by activated carbon.

B. Materials and Methods

1. Materials

Samples were acid-treated hydrolyzates with the highest fermentable sugar concentrations as determined from Chapter 4, which corresponded to three sulfuric acid concentrations (0.5, 1.25, and 2.0% H_2SO_4) obtained at 135ºC and 45 min with a final pH of 5-5.5.

Cellulase from *Trichoderma reesei* (Aqueous solution, ≥700 units/g) and ß-Glucosidase from almonds (lyophilized powder, 7.80 units/mg) were from Sigma-Aldrich (St. Louis, MO, USA). Activated carbon (Activated charcoal powder, USP grade) was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA).

2. Methods

Detoxification

Prior to enzymatic treatment, the three liquid fractions of acid-hydrolyzed SBM samples were separated from the solid fraction by centrifugation at 4500 RPM (Allegra X-22R centrifuge, Rotor SX4250, Beckman Coulter, Germany) for 35 minutes and 10ºC, followed by filtration using a Whatman #4 filter paper (110 mm Ø). Following the methods proposed by Buhner and Agblevor (2004), Carvalheiro et al. (2005) and Silva, et al. (1998), activated carbon was added to the liquid fraction (supernatant) for detoxification. Four different ratios of activated carbon were tested (0.5%, 1.0%, 1.5%, and 2.0%, w/v) for 1 h at room temperature (≈ 25ºC) without pH modification in an orbital shaker (Thermo Scientific Max Q 4450, Dubuque, Iowa, USA) set at 200 RPM. The activated carbon was removed from the liquid fraction by centrifugation at 4500 RPM—at the conditions established above for 20 min—and then filtrated using a Whatman #4 filter paper (110 mm Ø) (Buhner and Agblevor, 2004; Carvalheiro et al., 2005). Following

detoxification with activated carbon, the liquid fraction and the solid fraction were re-combined for enzyme hydrolysis (Figure 5.1).

Enzymatic hydrolysis of the acid hydrolyzed SBM

The cellulose, hemicellulose and some oligosaccharides remaining in the SBM after acid hydrolysis, with and without detoxification, were treated with Cellulase (E1), β-glucosidase (E2), and Cellulase + β-glucosidase (E3). The reaction consisted of 7 mL of acid-hydrolyzed sample (solid-liquid solution) + 3 mL of acetate buffer (pH 5-5.5) + 0.2 mL of each enzyme. Since β-glucosidase was in powder form, a 20 mg/mL solution of the enzyme was prepared in acetate buffer as described by the supplier. For the treatment with both enzymes (mix), 0.1 mL of each enzyme was combined to make a 0.2 mL solution.

Enzyme reactions were conducted at 50ºC and pH 5-5.5 for 20 hours in an orbital shaker at 200 rpm (Kim, et al., 1998). To inactivate the enzymes, the tubes were held for 5 minutes in a boiling water bath and then for 5 minutes in an ice water bath. The liquid fraction was separated from the solid part by centrifugation at 4500 RPM (Allegra X-22R centrifuge) for 35 minutes and 10ºC. Liquid samples were stored at -20 ºC prior to fermentable sugars analysis. Fermentable sugars were analyzed using the same HPLC method described in Chapter 3. The SBM solid fraction was dried at 60ºC for 24 h and stored at room temperature prior to cellulose analysis.

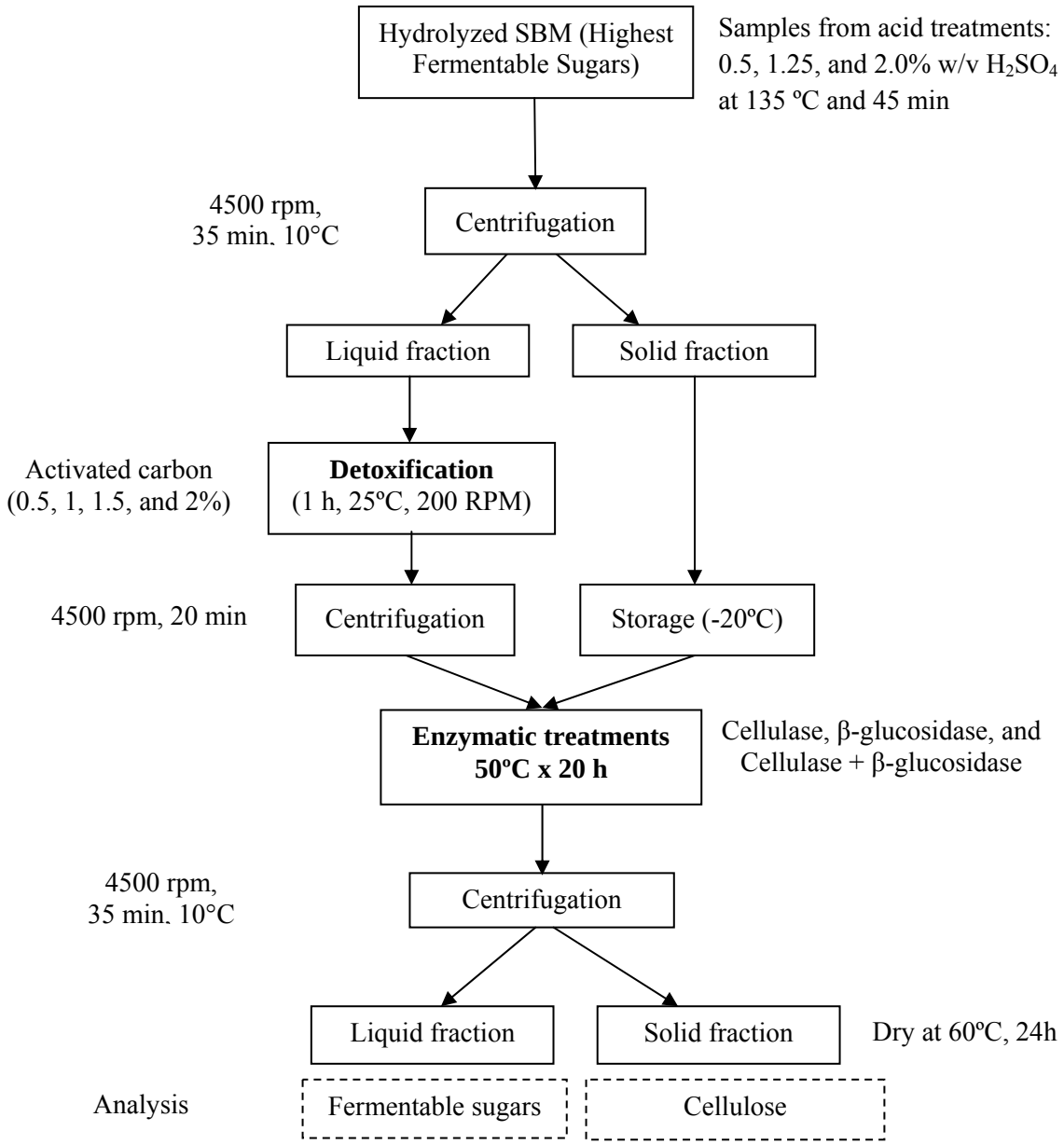


Figure 5.1. Detoxification with activated carbon followed by enzymatic hydrolysis with cellulase, ß-Glucosidase, and cellulase + β-Glucosidase of acid-hydrolyzed soybean meal

Note: After detoxification and previous to enzyme treatment the liquid and solid fractions were combined

Experimental design

For the enzymatic hydrolysis, the experimental design was a split-plot with a whole plot in a randomized complete block with the three enzyme types as factors. The split-plot portion was comprised of a substrate concentration - pretreatment factorial (with three substrate concentrations and two detoxification pretreatments). There were two replication of each treatment combination (Table 5.1).

To study the effect of different activated carbon ratios in the reduction of 5-HMF and furfural, experiments were arranged in a randomized completed block design with two factors (acid concentration and activated carbon ratio), two blocks, and two replications for each treatment combination.

Cellulose degradation in the hydrolyzed SBM

Cellulose degradation of samples subjected to cellulolytic enzyme treatment was evaluated by scanning electron microscope and the anthrone method (as described in Chapter 3).

Statistical analysis

For the enzymatic hydrolysis treatments and detoxification, as well as for cellulose degradation after cellulose treatments, data were analyzed with SAS Version 9.2 software. Analysis of variance (ANOVA) was carried out and the differences in the mean increase of fermentable sugar and 5-HMF and reduction of furfural were analyzed using the Fisher's least significant difference procedure ($\alpha = 0.05$).

Table 5.1. Experimental design for the enzymatic hydrolysis of soybean meal after acid hydrolysis

DAY	**1**			**2**			**3**			**4**		
RUN	1	2	3	4	5	6	7	8	9	10	11	12
Detoxification	ND	D	D	ND	D	D	ND	D	D	ND	D	ND
Enzyme	E1	E2	E2	E1	E3	E3	E1	E2	E1	E3	E2	E3
Substrate	S3	S1	S2	S2	S2	S1	S2	S3	S2	S2	S1	S3

DAY	**5**			**6**			**7**			**8**		
RUN	13	14	15	16	17	18	19	20	21	22	23	24
Detoxification	ND	D	ND	D	ND	ND	ND	D	D	D	D	D
Enzyme	E2	E1	E1	E1	E2	E3	E2	E3	E1	E1	E3	E1
Substrate	S3	S3	S1	S3	S1	S2	S2	S2	S1	S2	S3	S1

DAY	**9**			**10**			**11**			**12**		
RUN	25	26	27	28	29	30	31	32	33	34	35	36
Detoxification	ND	D	ND	ND	ND	D	ND	ND	ND	D	ND	D
Enzyme	**E3**	**E3**	**E1**	**E3**	**E3**	**E3**	**E2**	**E2**	**E1**	**E2**	**E2**	**E2**
Substrate	S3	S3	S1	S1	S1	S1	S2	S3	S3	S2	S1	S3

D: Detoxification (activated carbon 2% w/v, 1h at 25ºC)
ND: No detoxification,
E1: Cellulase
E2: ß-Glucosidase
E3: Cellulase + ß-Glucosidase
S1, S2, and S3: Samples from treatments with 0.5, 1.25, and 2.0% H_2SO_4 at 135ºC and 45 min (the highest fermentable sugar content observed in Chapter 3)

C. Results and Discussion

1. Detoxification of hydrolyzed SBM

Overall, in most treatments, the levels of 5-HMF and furfural were significantly reduced after treatment with activated carbon ($p < 0.0001$) and less than 1% of fermentable sugars were lost after the detoxification treatments. The highest reductions in 5-HMF and furfural were 90.20 ± 1.01% (mean ± S.E.) and 96.75 ± 0.85%, respectively, with the treatment using 2% activated carbon and hydrolyzate obtained with 0.5% H_2SO_4 at 135ºC and 45 min (shaded row in Table 5.2). Under the conditions evaluated in this study, activated carbon significantly reduced the levels of 5-HMF and furfural in hydrolyzed SBM; thus, hydrolyzates treated with activated carbon would be suitable substrates for the production of ethanol, butanol, xylitol, or lactic acid via fermentation due to the reduced levels of toxic compounds.

Overall, the best concentration of activated carbon for the reduction of 5-HMF and furfural was 2%, which is lower than 10% of activated carbon applied by Carvalheiro et al. (2005) in the detoxification of hydrolyzed brewery's spent grain. They reported only a 68% reduction in 5-HMF and 92% reduction in furfural. However, it was not an effective acetic acid detoxifier (only 17% reduction after treatment). Similarly, in this study the acetic acid retention, even following treatment with 2% activated carbon, was still 13.8 ± 3.9%; thus, acetic acid remained a potential inhibitor during fermentation. Hong et al. (2011) and Converti et al. (1999), both reported 95.4% retention of acetic acid with activated carbon (2%) in lignin derivatives (phenolics) after acid hydrolysis of wheat straw. Furthermore, Berson et al. (2005) reported that five-stage detoxification with activated carbon (8% w/v, 35°C) was necessary to reduce acetic acid by 88% in corn stover hydrolyzate.

Table 5.2. Means of final concentration of 5-HMF and furfural for different treatments with activated carbon of SBM samples treated at 135ºC for 45 min with variable concentrations of H_2SO_4

Treatments	HMF (mg/L)			Furfural (mg/L)		
Activated carbon (%)– H_2SO_4 (%)	Before	After	Reduction (%)	Before	After	Reduction (%)
0.5 - 0.5	0.20	0.09	55	30.50	8.84	71
0.5 - 1.25	0.90	0.60	33	246.60	133.16	46
0.5 - 2.0	1.80	1.40	22	320.70	208.46	35
1.0 - 0.5	0.20	0.04	78	30.50	3.05	90
1.0 - 1.25	0.90	0.35	61	246.60	71.51	71
1.0 - 2.0	1.80	0.97	46	320.70	125.07	61
1.5 - 0.5	0.20	0.03	87	30.50	0.92	97
1.5 - 1.25	0.90	0.21	77	246.60	39.46	84
1.5 - 2.0	1.80	0.72	60	320.70	86.59	73
2.0 - 0.5	0.20	0.02	90	30.50	0.92	97
2.0 - 1.25	0.90	0.14	85	246.60	27.13	89
2.0 - 2.0	1.80	0.47	74	320.70	54.52	83

LSD to compare 5-HMF means after treatments within the same column is 0.037 mg/L
LSD to compare furfural means after treatments within the same column is 5.12 mg/L

2. Enzymatic hydrolysis of the acid hydrolyzed SBM

Enzymatic treatments solely with cellulase were significantly different than the other treatments (Table 5.3) because β-glucosidase alone was not capable of degrading appreciable amounts of cellulose. Similarly, Hsu et al. (2011) reported that the activity of β-glucosidase in a commercial enzyme mixture was low as a result of low hydrolysis efficiency during the acid pretreatment of a corncob-based cellulosic material.

ß- glucosidase, which is in small quantities in the commercial cellulase mix, is a cellobiohydrolase that hydrolyzes cellobiose into two molecules of glucose, but normally it has low activity(Coughlan and Ljungdahl, 1988). Therefore, the minimal change in fermentable sugars after using solely ß- glucosidase is likely due to its inability to work on the partially hydrolyzed polysaccharide without pretreatment with cellulase. Whereas, the commercial cellulase also includes ß- glucosidase that could work after the intervention of the other enzymes present in the cocktail. Furthermore, Ollé et al (2000) reported—when working with polysaccharides in the substrate of cell wall of mango puree—partial inhibition of the ß-glucosidase activity that was attributed to glucose present at levels higher than 0.5%.

When cellulase was applied alone in the pretreated SBM, the most significant increase in fermentable sugars (12.34 g/L) (Table 5.3) was generated by the least severe pretreatment (T3C0t3). This can be explained by greater substrate availability for the enzyme cocktail. In contrast, with the more severe pretreatments, most of the substrates were already hydrolyzed by the acid pretreatment and the samples contained high levels of toxic compounds, such as acetic acid, that inhibited enzyme activity. This result is lower, but comparable, to the value (19.1 g/L glucose, xylose, and cellobiose) reported by Hsu et al. (2011) after treatment with cellulase following an acid pretreatment of a corncob-based cellulosic material.

Table 5.3. Increase in fermentable sugars after enzymatic treatments of hydrolyzed SBM pretreated at 135°C and 45 min with various concentrations of H_2SO_4.

		Increase in fermentable sugars (g/L)	
Enzyme	**Pretreated Substrate [H_2SO_4 (%)]**	**With detoxification**	**Without detoxification**
β-glucosidase (B)	0.0	0.21	0.03
	0.5	1.51	1.68
	1.25	4.10	2.40
	2.0	2.24	2.16
Cellulase (C)	0.0	12.34	2.81
	0.5	5.53	0.82
	1.25	6.42	0.59
	2.0	4.10	0.14
β-glucosidase + Cellulase (B+C)	0.0	6.34	6.23
	0.5	5.35	5.47
	1.25	4.63	1.03
	2.0	4.66	3.34

LSD to compare fermentable sugars increment means for different enzymes is 0.61 g/L
LSD to compare fermentable sugars increment means for different substrate-detox combination (at same enzyme) is 0.36 g/L

3. Cellulose degradation in the hydrolyzed SBM particles after enzymatic treatment

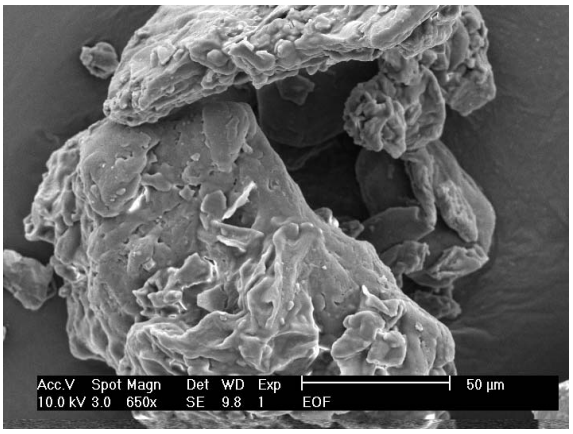
After the enzymatic treatment with cellulase, it was found that the final cellulose concentration was 1.74 % d.b when this enzyme was applied in the substrate obtained at 135°C, 0% H_2SO_4, 15 min, which is the less severe treatment (Table 5.4). A possible explanation is that cellulase worked more effectively following less severe acid pretreatment due to the lower concentration of inhibitors (5-HMF, furfural) from the pretreatment and activated carbon (2% w/v) treatment (that worked better on the lowest acid concentration). Less severe treatments may have also left enough material for the enzyme to act on. Furthermore, as Yoo et al. (2012) described in their work with soybean hulls, acid hydrolysis produces solubilization of hemicellulose which confirms that one of the substrates of the enzymatic action was not only cellulose but hemicellulose.

Images obtained by scanning electron microscope (Figure 5.2) were used as additional evidence of the enzymatic treatments effects on the structural carbohydrates to show the change in shape and size distribution of SBM particles. Clearly, there was degradation after acid hydrolysis in distinct levels depending upon the strength of the treatments. The surface particles are evidently different between treatments; surface particles from the untreated SBM (Figure 5.2a) have a smooth outer layer covering the surface probably comprised of cellulose, hemicellulose, lignin, and other binding materials. Similar observations were made by Corredor (2008). In contrast, the outer layer of the surface particles following enzymatic treatment (Figures 5.2b, 5.2c) appeared to be completely removed, and the particles are dispersed with smaller and irregular surfaces.

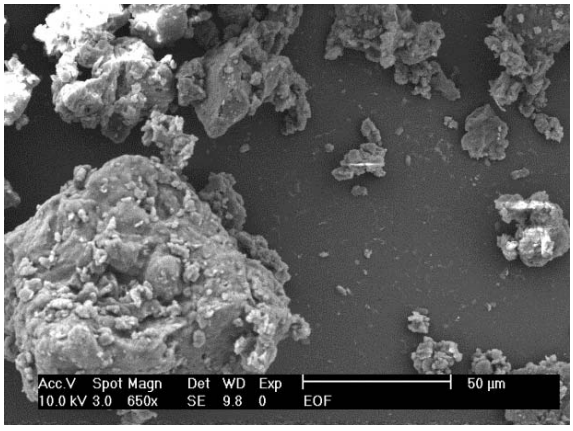
Table 5.4. Cellulose concentration after cellulase treatments of substrates pretreated at various concentrations of H_2SO_4 at 135^{o}C and 45 min.

Substrate [H_2SO_4 (%)]	**Cellulose (% d.b)**
0.0	1.74
0.5	2.24
1.25	2.09
2.0	2.64

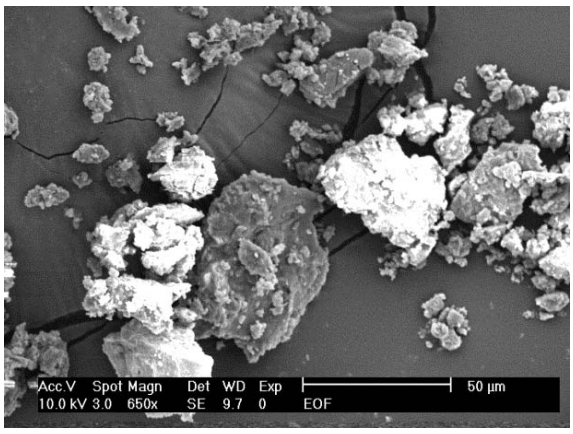
LSD to compare cellulose concentration means among treatments is 0.15 g/L



a)



b)



c)

Figure 5.2. Scanning electron microscope images of SBM particles. a) Untreated SBM, b) SBM after cellulase treatment of acid pretreated samples at 135°C, with 0.5% H_2SO_4 for 45 min, c) SBM after cellulase treatment at 135°C, with 2% H_2SO_4, for 45 min.

D. Conclusions

Among the enzymatic treatments applied to pretreated SBM samples, cellulase alone had the greatest effect on fermentable sugars (Δ=12.34 g/L). This increase in fermentable sugars was observed following a high temperature and long time pretreatment with no acid (135°C, 0% H_2SO_4, 45 min or T3C0t3), which is attributed to greater substrate accessibility during cellulase treatment. In the substrates subjected to more severe pretreatments, the major part of the substrates were already hydrolyzed by the acid, and unknown toxic compounds were likely still present causing inhibition in the enzymes.

Detoxification had a significant effect on enzyme performance due to the reduction of inhibitors such as 5- HMF and furfural. The enzymes were unable to work on most of the pretreated SBM without detoxification. The maximum reductions in 5-HMF and furfural were 90.20 ± 1.01% (mean ± S.E.) and 96.75 ± 0.85%, respectively, with the 2% activated carbon treatment with 0.5% H_2SO_4 at 135ºC and 45 min. Additionally, less than 1% of fermentable sugars were lost after the detoxification treatments.

CHAPTER VI

Quantification of nitrogen in the liquid fraction, and lysine bioavailability in solid fraction of SBM hydrolyzates

A. Introduction

After the acid and enzymatic treatments applied to SBM in Chapters 4 and 5, it was demonstrated that it is possible to obtain a liquid fraction rich in fermentable sugars and a solid fraction enriched in crude protein. It is hypothesized that in the liquid fraction, in addition to fermentable sugars, there is organic and inorganic nitrogen that could serve as nitrogen source for microbial fermentations. It is also likely that the acid and enzymatic treatment on the SBM could have improved amino acids (AA) availability, especially lysine that is the first limiting amino acid in diets (Chalova et al., 2007; Johnson, 1992). It has been demonstrated that higher amino acid availability, especially lysine, facilitates protein synthesis (Batterham, 1992; Lewis and Bayley, 1995), thus improving considerably the potential value of the SBM.

During the production of SBM; heat is applied to improve nutritional quality by inactivating anti nutritional factors, such as trypsin inhibitors (Liener, 1994), which in some situations, especially when high heat is used, can lead to reduced protein quality due to decreased amino acid availability or digestibility (Parsons et al, 1992). This decreased amino acid availability and the eventual formation of toxic compounds are in part due to the formation of Maillard reaction products that take place when proteins and glucose are heated together (Hurrell, 1990; Fernandez and Parsons, 1996; Erbersdobler et al., 1981, Johnson et al, 1977). On the other hand, low temperature treatments in an aqueous medium, as in the present research, can promote the solubility of nitrogen compounds and their production resulting from the denaturation of protein—in ammonia and other nitrogen forms—and increase the digestibility of

proteins, which could translate into an increased bioavailability (Barać et al., 2004; Wang and Johnson, 2001; Veličković et al., 1995).

After glucose and fructose as the main carbon sources, ammonium is the second most important nutritional factor as a nitrogen source in ethanolic fermentations (Snyder and Ingledew, 2012).Therefore, measuring the ammonium concentration available in the liquid fraction of SBM hydrolyzates is important for the formulation of the fermentation both. The increment of ammonium solubility and digestibility of soy proteins can increase after treatments at high temperatures and pressures (Barać et al., 2004).

In-vivo protein digestibility is the golden standard to estimate AA availability. However, these approaches are expensive and time consuming. A more straight forward method is the use of in-vitro assays, especially for preliminary screening studies of large numbers of samples. The basic approach for in-vitro tests is the digestion of the protein with a cocktail of enzymes, similar to the ones found the gastric juices, followed by determination of amino acids by different methods (Chalova et al., 2007, Stein et al., 2007). One of the detections methods is the use of *Escherichia coli* as biosensor, which has been reported to be effective in terms of time, cost, and consistency (Erickson et. 2000).

Traditionally, measures of in-vivo digestibility have been used to estimate AA bioavailability (Sauer and Ozimek, 1986). Currently, there are others methods available, such as AA digestibility and microbiological (biosensor) assays, which are more suitable for estimating AA bioavailability than the traditional assay (Chalova et al., 2007, Stein et al., 2007). Among them, microbiological assays for AA bioavailability, such as *Escherichia coli* biosensor, are more effective in terms of time, cost and variability (Erickson et al., 2000).

The goal of this Chapter was to quantify total nitrogen and ammonium in the liquid fraction of hydrolyzed SBM and to evaluate total and bioavailable lysine by whole-cell biosensor in the solid fraction of the hydrolyzed SBM.

B. Materials and Methods

1. Materials

Bacteria used in the bioavailability experiments was the *gfpmut3* containing lysine auxotroph strain *E.coli* ΔlysA mini-Tn5-Km-gfpmut3 (-800C) (Lys. biosensor XL 329) which was obtained from the Biomass Research Center at the University of Arkansas (Fayetteville, Arkansas. USA). The strain was stored at 4°C on Luria Bertani (LB) agar medium supplemented with filter sterilized ampicillin (100 μg/ml) and kanamycin (50 μg/ml) (Chalova et al., 2007). Samples used in this research were originated from Chapters 4 and 5 and are shown in Table 6.1.

2. Methods

Quantification of total nitrogen and ammonium nitrogen in the hydrolyzate liquid fraction

Total nitrogen concentration in the hydrolyzate liquid fraction was determined by the nitrogen combustion method (A.O.A.C., 1990b) using an Elementar Variomax Instrument (Elementar Americas, Inc. Mt. Laurel, NJ, USA). Ammonium nitrogen was analyzed with a Skalar Sanplus Autoanalyzer (Skalar, Inc., GA, USA) by the salicylate color method based on the modified Berthelot reaction. Briefly, ammonia was chlorinated to monochloramine, which reacts with salicylate to 5-aminosalicylate. After oxidation and oxidative coupling, a green color complex is formed, which is measured at 660 nm. Samples were conveniently diluted and fed into the machine at a flow of 0.16 ml/minute during 5 minutes each run (Krom, 1980; Searle,1984).

Quantification of total lysine by HPLC in the solid fraction

Solid SBM samples were hydrolyzed with 6 N HCL, as it was described by Fountoulaki and Lahm (1998). Aliquots of 0.5 g were suspended in 5 mL of 6N HCL in glass tubes. After purging the head spaces with Nitrogen gas, the tubes were capped and incubated at 110°C for 24 h in a bench top oven. The hydrolyzates were subsequently neutralized with potassium hydroxide to pH 7, filtered through Whatman No. 4 filter paper, and diluted with distillated water to 50 mL. The samples were stored at -20°C until further analysis.

Lysine total content was analyzed by HPLC after derivatization with OPA (ortho-phthalaldehyde) according to the method by Henderson et al. (2000). The HPLC was a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of binary pumps, auto sampler, column oven, UV-Vis detector, and degasser. The separation was made in a reverse-phase Zorbax Eclipse AAA column (4.6 x 150 mm) maintained at 40°C with a binary gradient consisting of 40 mM sodium phosphate buffer (NaH_2PO_4), pH 7.8 (eluent A) and acetonitrile:methanol:water (45:45:10 v/v/v) (eluent B) at a flow rate of 2 mL/min. The gradient program was the following: 0%B from 0 to 1.9 min, a linear gradient to 76% B from 1.9 to 20 min, a linear gradient to 100% B from 20 to 21 min, 100% B from 21 to 24 min., and a linear gradient to 0% B from 24 to 28 min. The injection volume was 10 µL and detection was performed at 338 nm. Lysine was identified and quantified using a standard curve made with lysine, 99.5% purity, from Fluka Analytical (Sigma Aldrich, St. Louis, MO).

Preparation of SBM enzymatic hydrolyzates for the bioavailability test

The procedure describe by Chalova et al. (2007) was followed to obtain the enzymatic hydrolyzate samples for the bioavailability test. Samples of 5 mg of finely ground SBM were added to 5ml of 0.1M potassium phosphate buffer, pH 6.8, in 15-ml screw-cup centrifuge tubes.

Protease from *Streptomyces griseus* (Sigma Aldrich, St Louis, MO) and MicroScan® peptidase Reagent B1012-30B-30 peptidase (Siemens Healthcare Diagnostics Inc, Deerfield, IL) were added to a final concentrations of 1.0 and 0.5 mg/ml respectively. Tubes were vortexed and placed in a VWR Model 1227 water shaking bath (VWR Radnor, PA) set at 37^{o}C and 150 strokes/min and allowed to digest for 4 h. At the end of the digestion, digestates were placed in a water bath at 100^{o}C for 15 min and then into an ice bath for 5 min to inactivate the enzymes. The digestates were centrifuged for 20min at 3000 x g and 10^{o}C, filter sterilized, and the permeates collected and saved at -20^{o}C for biosensor lysine assays.

Quantification of total lysine and bioavailable lysine by *E. coli* biosensor

The *E. coli* used as biosensor was grown overnight at 37^{o}C in 5 ml LB broth containing ampicillin and kanamycin antibiotics in a Chest Type New Brunswick Scientific Controlled Environment Incubator Shaker, Model G-25, (New Brunswick Scientific, Enfield, CT) set at 180 strokes/min. Then, the bacterial suspension was diluted with LB broth until the optical density at 420 nm (OD 420) reached 0.5 units of absorbance. Aliquots of 150 µl of bacterial culture were transferred into 5 ml Davis minimal medium (without lysine) to deplete the endogenous lysine of the bacterial cells, then incubated for 10 h, at 37^{o}C, in the shaker bath as described above. After 10 h, the culture was diluted with enough Davis medium to achieve 0.4 units of absorbance at 420 nm. Aliquots of 150 µl of culture were inoculated to 5 ml of test medium Davis + antibiotics (ampicillin and kanamycin) + lysine (in appropriate amounts to have final concentrations of 0, 3, 6, 10, and 15 ug/ml) + galactose + IPTG. The tubes were incubated at 37^{o}C and 180 strokes/min as it was described previously. The OD was measured after 24h, which was the time when the maximum optical density was reached, for each lysine concentration to construct a calibration curve of OD versus lysine concentration.

The bioavailability of lysine was calculated as: (A/G)*100; where A (% d.b) was the amount of lysine determined with the biosensor and G (% d.b) was the concentration of lysine determined by HPLC (Chalova et al. 2007).

Experimental design

To study the effect of different treatments on total nitrogen and ammonium in the hydrolyzate liquid fraction, experiments were arranged in a randomized complete design with three factors (acid concentration, cellulase, β-glucosidase) and two replicates for each treatment. The effect of different treatments in the lysine bioavailability of the hydrolyzate solid fraction were studied with randomized complete design with two factors (acid concentration, enzyme treatment) and two replicates for each treatment.

Statistical analysis

For the total nitrogen and ammonium in the hydrolyzed liquid fraction and for the lysine bioavailability in the solid fraction (Tables 6.2 and 6.3), data were analyzed with a three-way analysis of variance and the LSD Test ($\alpha = 0.05$) using SAS Version 9.2 software (SAS institute Inc., Cary, NC, USA).

Table 6.1. Factors and levels used for a one-way ANOVA to analyze total nitrogen and ammonium in the hydrolyzates liquid fraction, and lysine and lysine bioavailability in the solid fraction

Total Nitrogen and ammonium nitrogen	**Total lysine and lysine bioavailable lysine**
T3C0t3 + Cellulase	T3C0t3 + (Cellulase + ß-glucosidase)
T3C1t3 + Cellulase	T3C1t3 + (Cellulase + ß-glucosidase)
T3C2t3 + Cellulase	T3C2t3 + (Cellulase + ß-glucosidase)
T3C3t3 + Cellulase	T3C3t3 + (Cellulase + ß-glucosidase)
T2C2t2 + Cellulase	T2C2t2 + (Cellulase + ß-glucosidase)
T3C0t3 + β-glucosidase	T3C0t3 + Cellulase
T3C1t3 + β-glucosidase	T3C1t3 + Cellulase
T3C2t3 + β-glucosidase	T3C2t3 + Cellulase
T3C3t3 + β-glucosidase	T3C3t3 + Cellulase
T2C2t2 + β-glucosidase	T2C2t2 + Cellulase
T3C0t3 +(Cellulase + ß-glucosidase)	No treatment
T3C1t3 + (Cellulase + ß-glucosidase)	T3C0t3
T3C2t3 + Cellulase + ß-glucosidase)	T3C1t3
T3C3t3 + (Cellulase + ß-glucosidase)	T3C2t3
T2C2t2 + (Cellulase + ß-glucosidase)	T3C3t3
T3C0t3	T2C2t2
T3C1t3	
T3C2t3	
T3C3t3	
T2C2t2	

T2= 120°C (15PSI), T3= 135°C (32PSI)
C0=0%, C1=0.5%, C2=1.25%, C3=2% H_2SO_4
t2= 30 min, t3= 45 min

C. Results and Discussion

1. Total nitrogen and ammonium nitrogen in the hydrolyzate liquid fraction

For hydrolyzates obtained with the same H_2SO_4 concentrations, most enzymatic treatments did not increase significantly either total nitrogen or ammonium nitrogen in the liquid fraction (Table 6.2, comparing the same H_2SO_4 concentrations among treatments). But, total nitrogen and ammonium analysis indicated that nitrogen compounds in the liquid fraction increased more with treatments under high sulfuric acid concentrations (1.25% and 2%) than with lower concentrations (0% and 0.5%) or with the treatment T2C2t2 (120°C , 1.25% H_2SO_4, 30 min.). Previously, Barać et al., (2004) showed that the solubility and digestibility of soy proteins increase after treatments at high temperatures and pressures. Heating soy proteins above 70°C causes dissociation of their quaternary structures, denatures their subunits, and promotes the formation of protein aggregates via electrostatic, hydrophobic and disulfide interchange mechanisms (Barać et al., 2004). Veličković et al. (1995) demonstrated that high content of soluble glycinin, the main lysine-containing globulin protein and one of the main reserve proteins in soy beans, was found in the soybean treated after 45 minutes of moist steaming at 2.0 bars (29 PSI), which are conditions very close to the one applied in this research.

Watanabe et al. (1974) conducted experiments with acid hydrolysis of defatted soy proteins—with 18% HCl followed by neutralization—to obtain hydrolyzed vegetable protein. These treatments increased the solubility of soybean proteins. This latter research also suggested the use of acid treatment (pH 2-3) in combination with thermal treatment where the increase of the solubility was explained due to the partial deamination and mild hydrolysis (Watanabe et al., 1974. However, highest increase in protein solubility is only achieved at considerably low pH,

high temperatures and long incubation times (Matsudomi et al.,1985) as it was developed in our research.

After the main carbon sources (glucose and fructose), ammonium is the most important nutritional factor in ethanolic fermentations for cell growth, cell maintenance, and ethanol production (Snyder and Ingledew, 2012). The concentration of ammonium regularly is 0.3 to 0.5 g/L to produce ethanol (Wang et al., 2012; Mullins and Nesmith, 1987).The ammonium concentration in this research varied from 0.20 to 1.24 g/L (Table 6.2), which indicates that the hydrolyzate liquid fraction has enough nitrogen to support ethanolic fermentations and other bioprocesses having similar nitrogen needs.

Table 6.2. Means of total nitrogen and ammonium in the SBM liquid fraction after acid and enzymatic treatments

Treatment	**Total Nitrogen (g/L)** *SE=0.2070*	**Ammonium (g/L)** *SE=0.02676*
T3C0t3	6.23	0.22
T3C1t3	4.93	0.17
T3C2t3	8.72	0.73
T3C3t3	10.37	1.10
T2C2t2	4.88	0.31
T3C0t3 + Cellulase	6.97	0.21
T3C1t3 + Cellulase	6.74	0.23
T3C2t3 + Cellulase	9.32	0.80
T3C3t3 + Cellulase	10.46	1.24
T2C2t2 + Cellulase	5.87	0.28
T3C0t3 + β-glucosidase	6.55	0.22
T3C1t3 + β-glucosidase	5.91	0.20
T3C2t3 + β-glucosidase	10.14	0.76
T3C3t3 + β-glucosidase	10.97	1.24
T2C2t2 + β-glucosidase	5.36	0.24
T3C0t3 + (Cellulase + ß-glucosidase)	6.55	0.27
T3C1t3 + (Cellulase + ß-glucosidase)	6.11	0.21
T3C2t3 + (Cellulase + ß-glucosidase)	10.80	0.75
T3C3t3 + (Cellulase + ß-glucosidase)	10.74	1.16
T2C2t2 + (Cellulase + ß-glucosidase)	5.39	0.27

T2= 120°C (15PSI), T3= 135°C (32PSI)
C0=0%, C1=0.5%, C2=1.25%, C3=2% H_2SO_4
t2= 30 min, t3= 45 min
LSD to compare Total Nitrogen among treatments is 0.432
LSD to compare Ammonium among treatments is 0.056

2. Quantification of total lysine and bioavailable lysine by whole-cell biosensor

Figure 6.1 shows the lysine calibration curve obtained with the *E.coli* biosensor after 24 hours of growth for lysine concentrations of 0, 3, 6, 10, and 15 ug/ml. Lysine bioavailability (%) in the untreated SBM used for this experiment was 82 % (Table 6.3, column 4), which is relatively higher than the 70% reported by Erickson et al. (2000) and close to 92% reported by Chalova et al. (2007) using a similar biosensor. In this research, when the untreated SBM is compared with the treated ones, it becomes apparent that all treatments improve the lysine bioavailability ($p<0.0001$). The minimum average lysine bioavailability value was 92%, after treatment of 120°C, 1.25% H_2SO_4, 30 min (T2C2t2), which is in within the range from 95.2 to 97.2% reported by Cortes-Cuevas et al. (2011). The remainder of the treatments displayed lysine bioavailability values between 93 and 97%. These results demonstrated that under the conditions applied in this research the lysine bioavailability increased significantly and was minimally affected by the more severe acid hydrolysis conditions or by the enzymes. Fernandez and Pearson (1996) found that bioavailability of lysine in SBM autoclaved at 114°C for 40-60 min. was in the range of 81 to 87% and decreased when the severity in the heat treatment increased.

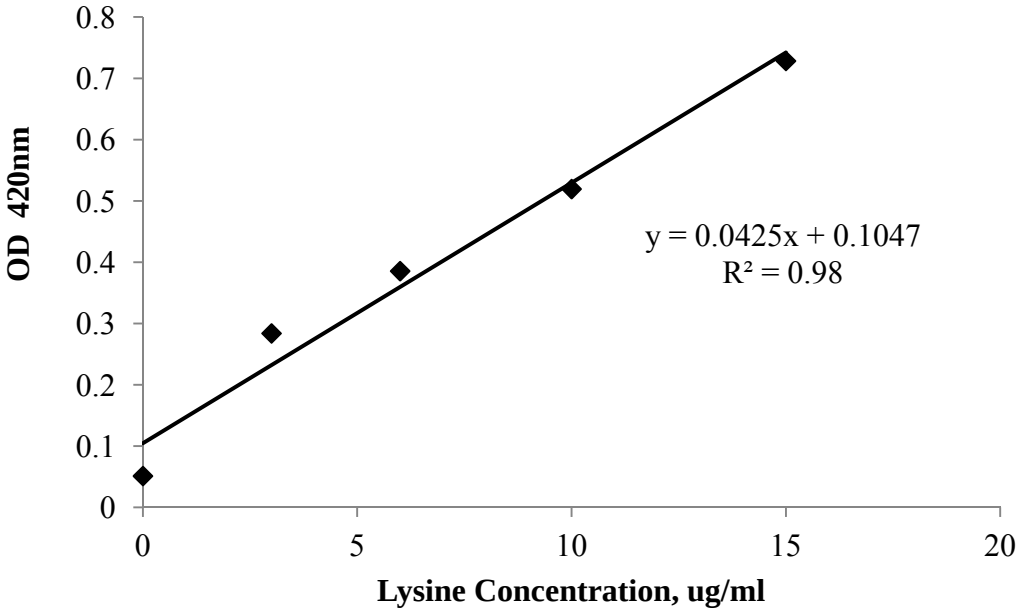


Figure 6.1. Lysine calibration curve obtained with the biosensor *E.coli* ΔlysA mini-Tn5-Km-gfpmut3 (-800C) (Lys. biosensor XL 329)

Table 6.3. Bioavailability of lysine in hydrolyzed SBM samples after enzymatic and acid hydrolysis.

Treatment	Lysine content (% d.b.)		Lysine bioavailability by biosensor
	By biosensor	By acid digestion and HPLC	
	SE=0.0325	*SE =0.0667*	*SE = 0.01304*
T3C0t3	3.75	4.12	93.70
T3C1t3	3.65	3.94	93.47
T3C2t3	3.68	3.83	93.09
T3C3t3	3.19	3.37	94.07
T2C2t2	3.75	3.88	94.98
T3C0t3 + (Cellulase + ß-glucosidase)	3.69	3.84	97.01
T3C1t3 + (Cellulase + ß-glucosidase)	3.62	3.83	95.13
T3C2t3 + (Cellulase + ß-glucosidase)	3.44	3.60	95.46
T3C3t3 +(Cellulase + ß-glucosidase)	3.09	3.25	96.59
T2C2t2 + (Cellulase + ß-glucosidase)	3.31	3.58	92.06
T3C0t3 + Cellulase	3.76	4.02	93.93
T3C1t3 + Cellulase	3.71	3.93	95.00
T3C2t3 + Cellulase	3.58	3.67	96.73
T3C3t3 + Cellulase	3.25	3.51	93.02
T2C2t2 + Cellulase	3.79	3.97	94.62

T2= 120°C (15PSI), T3= 135°C (32PSI)
C0=0%, C1=0.5%, C2=1.25%, C3=2% H_2SO_4
t2= 30 min, t3= 45 min.
For untreated SBM: Lysine by biosensor = 2.96 %, Lysine by acid digestion and HPLC = 3.64%, Bioavailability = 82.0
LSD to compare means of lysine bioavailability is 0.028.

The ANOVA performed on the concentration of lysine in samples analyzed by the biosensor (Table 6.3, column 1), showed significant differences among treatments ($p<0.0001$). The average untreated SBM lysine content was 2.96% d.b, which is comparable to 3.1% in SBM reported by Fernandez and Pearson (1996) and 3.02% reported by Chalova et al. (2007). The maximum value reached in this experiment was 3.79% d.b. for the treatment T2C2t2 + cellulase , which did not show significant differences with the untreated SBM, and the minimum 3.09% d.b for the treatment T3C3t3-Cellulase + ß-glucosidase. These results demonstrated that SBM, either untreated or treated, are both good sources of the essential amino acid lysine.

The total lysine concentration in SBM samples analyzed by HPLC with previous enzymatic digestion is also displayed in Table 6.3 (column 3). The ANOVA revealed significant differences among treatments ($p<0.0001$). The average of untreated SBM total lysine concentration was 3.43% d.b, which is comparable to 3.02% in SBM found by Chalova et al. (2007); while the highest value was 3.85% d.b. for the sample treated according to T2C2t2 and the lowest 3.32% d.b was reached for the treatment T3C3t3-cellulase + ß-glucosidase. These results are very similar to those obtained by the biosensor, thus demonstrating than the biosensor produces comparable result with HPLC.

The total lysine concentration in SBM samples analyzed by HPLC after digestion with HCl is shown in Table 6.3 (column 2). The ANOVA indicates significant differences among treatments ($p<0.0001$). The average untreated SBM total lysine concentration was 3.64% d.b, which is close to 3.26% d.b in SBM reported by Chalova (2007), and 3.3% d.b reported by Awawdeh et al., (2008); but over the range 2.5 to 2.7 % d.b of total lysine reported by Grieshop et al. (2003). The highest average value reached by treatments was 4.12% d.b. by T3C0t3 (135°C, 45 min., 0% H_2SO_4) and the minimum 3.25% d.b was reached by C3Mix (135°C, 45

min., 2% H_2SO_4 and mix of cellulase + ß-glucosidase), which is lower than the original because of the severe acid hydrolysis conditions. Taking HPLC results as control of measure, it is possible to establish that biosensor and HPLC analysis of lysine previous digestion with peptidase and protease result in inferior lysine concentrations due to the protein hydrolysis was not completed. However, the results obtained are very comparable and reliable among the data obtained in this research and with other studies such as Awawdeh et al., (2008), Chalova (2007), Grieshop et al. (2003), and Ericson et al. (2000).

D. Conclusions

After the dilute acid hydrolysis with H_2SO_4 and enzymatic hydrolyses with cellulase plus ß-glucosidase, liquid fraction of SBM reached ammonium concentrations between 0.20 and 1.24 g/L which is a significant improvement to generate a hydrolyzed SBM liquid fraction that could serve as potential substrate for ethanolic fermentation and other bioprocess. Lysine bioavailability in hydrolyzed SBM solid fraction increased in all treatments after dilute acid and enzymatic hydrolyses applied in this research. The major increase in lysine bioavailability was from 82% d.b to 97% with the treatment at 135°C, 45 min., 0% H_2SO_4, plus a mix of cellulase + ß-glucosidase. These results also suggest that a successful treatment in terms of profitability can be applied without enzymatic hydrolysis at low acid concentrations, which will depend of the further applications for this bio-product.

CHAPTER VII

Bioethanol production with the liquid fraction of SBM hydrolyzate as a substrate

A. Introduction

Saccharomyces cerevisiae is without doubt the most common microorganism used in the industrial ethanolic fermentation. This yeast is among the best known cells; additionally, it is highly robust, very resistant to toxic inhibitors and grows well at low pH, which minimizes the risk of contamination (Weber et al., 2010). On the other hand, *Z. mobilis* is a Gram negative bacterium that has been attracting abundant attention in the ethanol fuel production due to its high productivity. However, it has low resistance to toxic inhibitors and it can ferment only glucose, fructose, and sucrose (Weber et al., 2010; Doran, 1997; Rogers et al., 1982).

Currently, the production of renewable fuels, such as bioethanol, obtained from agricultural residues is gaining in importance. Even though a large volume of this fuel is produced from sugar cane sucrose and beet, bioethanol production from alternative sources can be attractive, especially when produced as a co-product associated with existing industries (Neureiter, et al., 2002). Bioethanol produced from lignocellulosic material is considered a renewable option that may improve the local production of fuels, reactivate rural economics, and reduce pollution (U.S. Department of Energy, 2010b). Bioethanol is one of the most promising and sustainable fuels and the interest for lignocellulose material have been increasing in recent years because of its low price and it is a widespread carbon resource (Chen et al., 2009). Among the crop options, soybean meal (SBM) can be a good alternative to produce bioethanol by taking advantages of its high carbohydrate content while, at the same time, its high protein content and quality could be still used for the animal feed industry; so it would not compete with food supplies, as occurs with

corn, sugarcane and beet. However, before SBM could be used as a substrate, it needs to undergo an acid hydrolysis—or other type of hydrolysis—to release fermentable sugars from the solid matrix.

During acid hydrolysis of materials containing lignocellulose, like the case of SBM, some inhibitory compounds such as 5-HMF, furfural, and acetic acid can be produced and become inhibitors of the ethanolic fermentation (Chen et al., 2009; Alves et al., 2002; Davis et al., 2005). Phenols, furans, carboxylic acids, and salts, are fermentation inhibitors with negative effect on cell membrane function, growth, and glycolysis in ethanol-producing yeast and bacteria (Klinke et al., 2004).

In order to eliminate or reduce these inhibitors considerable of efforts have been made, including optimizing operation parameters for lignocellulose degradation and fermentation (Carvalheiro et al., 2004), screening inhibitor-tolerant strains (Chen et al., 2009), and removing the inhibitors from the lignocellulose hydrolyzate (De Mancilha and Karim, 2003). Yeast can tolerate certain concentrations of furfural and acetic acid present in the substrate and it can even be converted to other less inhibitory compounds during the fermentation by yeast (Carvalheiro et al., 2004, Horvath et al., 2003); nonetheless, the effects of a certain lignocellulose degradation inhibitors for different strains and bacteria may be different (Keating et al., 2006; Larsson et al., 1999) depending of the mechanisms for which was attributed to the different influence on carbon metabolism (Hristozova et al., 2008; Gorsich et al., 2006; Lin et al., 2009) and nitrogen assimilation (Hristozova et al., 2008).

The minimum inhibitory concentration (MIC) is the lowest concentration of a toxic compound that will inhibit the detectable visual growth of a microorganism after overnight incubation. MICs are used by diagnostic laboratories mainly to confirm resistance, but most

often as a research tool to determine the in vitro activity of new antimicrobials, (Andrews, 2001). MICs are considered the "gold standard" for determining the vulnerability of organisms to toxic compounds and are used to evaluate the performance to all other methods of susceptibility testing (Andrews, 2001).

In this research MIC was used to evaluate the effect of inhibitors present in the liquid fraction of hydrolyzed SBM. It was possible to identify which SBM broth could be considered as an appropriate or harmful substrate for the yeast and bacteria applied to produce bioethanol. The main goal was to evaluate the capability of *S. cerevisiae and Z. mobilis* to produce ethanol using as substrates SBM hydrolyzates obtained according to the methods developed in Chapter 4.

B. Materials and Methods

1. Materials

The strains *S. cerevisiae* (NRRL Y-2233) and *Z. mobilis* subspecies mobilis (NRRL B-4286) were provide as a lyophilized powder by the ARS culture collection of the United States Department of Agriculture, Agriculture Research Service (Preoria, IL, USA). YM agar and YM broth were from Becton, Dickinson, and Company (Sparks, MD, USA). Furfural (99%) was acquired from TCI America (Portland, OR, USA), 5-HMF (99%) from SAFC Supply Solutions (St. Louis, USA), and acetic acid (97.7%) from VWR (West Chester, PA, USA).

***Substrates for fermentation*:** The substrates for the ethanolic fermentation were the hydrolyzates with the highest fermentable sugar concentration after acid and enzymatic hydrolysis obtained in Chapter 4. These were four hydrolyzates obtained after treatments at 135 ºC and 45 min for four acid concentrations (0.0, 0.5, 1.25, and 2.0% H_2SO_4) plus and additional SBM hydrolyzate obtained at 120ºC, 1.25% H_2SO_4, 30 min (T2C2t2), which was selected

because it showed a good balance between high sugar and protein content. These substrates will be called from now on soybean meal broth (SBMB) as is shown in Table 7.1.

2. Methods

Tolerance of *S. cerevisiae* and *Z. mobilis subspecies mobilis* to inhibitory compounds

The effects of furfural, 5-HMF, and acetic acid on cultures were evaluated using the (SBMB), and synthetic YM broth composed of glucose,10 g/L; yeast extract, 3 g/L; peptone, 5 g/L; and malt extract, 3 g/L, which were spiked with varying concentrations of these inhibitory compounds either alone or in combinations of the three (Table 7.1). The inhibitory compounds concentrations were chosen based on the literature (Da Cunha-Pereira et al., 2011; Klinke et al., 2004; Taherzadehet al., 2000) and were deliberately higher than their concentrations in SBM hydrolyzate to effectively gauge cell tolerance towards these compounds. The same medium without addition of the inhibitory compounds was used as control. Cultures were carried out in 15 mL sterile tubes containing 8 mL of YM broth with an initial pH 5.5 and incubated at 30ºC and 180 rpm for 24 h in an orbital shaker Thermo Scientific Max Q 4450 (Thermo Scientific, Dubuque, Iowa, USA). The inoculum was made by adding 0.8 mL (1% v/v) of the culture (10^6 cell/ml) from *S. cerevisiae or Z. mobilis* by using the bioreactor peristaltic pump (Bioflo/Cellin Gen 115 Benchtop fermentor and Bioreactor, New Brunswick scientific; Edison, New Jersey, USA). Samples were taken after 24 hours to read visual turbidity in the tubes (for the synthetic medium) and viability of cells by microscopy with Trypan blue staining (for the SBMBs) which are evidences of growth (+) or absence of growth (-). All the experiments were carried out in duplicate.

Table 7.1. Assay to test the tolerance to inhibitory compounds of *S. cerevisiae* (NRRL Y-2233) and *Z. mobilis* subspecies *mobilis* (NRRL B-4286)

	Concentrations of Inhibitors (g/L)			
Tube #	**5-HMF**	**Furfural**	**Acetic acid**	**Inhibitors Mix (5-HMF/Furfural/Acetic acid)**
1	0	0	0	0/0/0
2	0.1	0.1	2	0.1/0.2/0.2
3	0.2	0.2	4	0.2/0.3/0.4
4	1	1	5	1/1/2
5	2	2	6	2/2/4
6	3	3	7	3/3/6
7	4	4	8	4/4/8
8	5	5	9	5/5/10
9	6	6	10	6/6/12
10	7	7	11	7/7/14
			Obtained by acid hydrolysis at:	
11	SBMB0		135ºC, 0 % H_2SO_4, 45 min	
12	SBMB1		135ºC, 0.5% H_2SO_4, 45 min	
13	SBMB2		135ºC, 1.25% H_2SO_4, 45 min	
14	SBMB3		135ºC, 2% H_2SO_4, 45 min	
15	SBMB4		120ºC, 1.25% H_2SO_4, 30 min	

SBMB (Soybean meal broth)

Bioethanol fermentation with *Saccharomyces cerevisiae* and *Zymomonas mobilis*

Cells reactivation: *S. cerevisiae* and *Z. mobilis* were reactivated in 5 mL of yeast malt (YM) and incubated at 30ºC for 24h in an orbital shaker (Thermo Scientific Max Q 4450, Dubuque, Iowa, USA) at 150 rpm.

Preinoculum and inoculum: The pre-inoculum medium consisted of 5 mL of sterile YM broth at 30 ºC where both *S. cerevisiae* and *Z. mobilis* were inoculated separately and grown overnight in the orbital shaker. Then, for each strain, 0.2 mL of culture was plated by spreading out with a loop over the surface of two Petri dishes containing YM agar composed of 20 g/L of glucose, 3 g/L of yeast extract, 5 g/L of peptone, 3 g/L of malt extract, and grown overnight at 30 ºC before added to the final inoculum. The inoculum was prepared by adding all the colonies formed in both dishes to 80 ml of SBMB, which was10% of the 800-ml total fermentation volume, and allowing the cells to adapt by incubating overnight at 30 ºC in an orbital shaker.

***Fermentation*:** Fermentations were conducted at 30ºC for 36 h with an initial pH of 5-5.5, a percentage of dissolved oxygen (%DO) less than 1%, after stabilization, and an initial biomass concentration between 7×10^6 and 1×10^7 cells/mL (Laopaiboon et al., 2009 and Siqueira et al., 2008, Mullins and Nesmith, 1987). The fermentor was a 1.3-liter Bioflo/CellinGen 115 Benchtop Fermentor & Bioreactor (New Brunswick scientific; Edison, New Jersey, USA) with temperature, pH, %DO, agitation, pump feed, antifoam, and level control. The glass vessel head plate had an inoculation port and wells for a resistance temperature detector (RTD), a foam probe, a sparger, a harvest tube, a sampling tube, an exhaust condenser, a DO probe, and a pH electrode. Samples during the fermentation were taken every 4h to estimate fermentation the kinetic parameters: biomass yield from sugar (Yx/s), ethanol yield from sugar (Yp/s), and volumetric ethanol productivity, rp (g/L/h); where x is the biomass (live cells), p is the product

(ethanol) and s is the substrate (sugars). Cell density (number cells/L) was determined with a hemacytometer (Neubauer chamber) placed on a phase contrast microscope (Nkon Eclipse E400, Japan). The cell density was correlated with a calibration curve of cell dry weigh obtained by drying aqueous solutions of known concentrations of cells at 80ºC for 20-24 hours (Buhner, and Agblevor, 2004, Alfenore et al., 2002). All kinetic parameters were calculated using the following equations:

$Yx/s = -\frac{dx}{ds}$ Biomass yield (g biomass / g sugars) (7.1)

$Yp/s - \frac{dp}{ds}$ Ethanol yield from substrate (g ethanol / g sugars) (7.2)

$rp = (qp)x$ Volumetric ethanol productivity (g ethanol / Lh) (7.3)

$qp = \left(\frac{dp}{dt}\right)\left(\frac{1}{x}\right)$ Specific rate of product formation (7.4)

$ms = \frac{\left[(rs) - \frac{rx}{Yxs} - \frac{rp}{Yps}\right]}{x}$ Maintenance coefficient (g sugar/g cell h) (7.5)

Where,

rs = g sugar consumed/L h

rx = g cell produced/Lh

rp = g ethanol produced/Lh

Growth kinetics and model development for the ethanol fermentation of hydrolyzed SBM broths

By integration of Logistic Model equation (Eq. 2.2 in Chapter 2), the kinetic of biomass production rate can be calculated with the following equation:

$$X = \frac{X_0\, X_{max}\, e^{\mu_{max} t}}{X_{max} - X_0 + X_0 e^{\mu_{max} t}}$$

This equation relates biomass (X) production and the fermentation time (t) and was used to fit the experimental data. For the calculation of the kinetics parameters only glucose and fructose were considered as growth-limiting-substrates because these sugars had the highest and consistent consumption by the microorganisms.

Experimental design

For the fermentation, the experimental design was a complete randomized block design with microorganism type and substrate concentration as factors. Experiments were run and analyzed by replication (Table 7.2).

Statistical analysis

Data were analyzed with SAS Version 9.2 software (SAS institute Inc., Cary, NC, USA) to determine the kinetic parameters μ_{max}, $X_{max,}$ and X_0. Analysis of covariance (ANCOVA) was used to fit the data to the logistic model using the Gauss-Newton non-linear regression method and comparisons of each regression coefficient across the treatments. For the minimum inhibitor concentrations and kinetics parameters, data were analyzed with JMP® version 9.0.0 (SAS institute Inc., Cary, NC, USA). To analyze the kinetic parameters (Yp/s, Yx/s, ms, and qp) analysis of variance (ANOVA) was also carried out and differences in the mean of the kinetic parameters were analyzed by Tukey-Kramer test ($\alpha = 0.05$).

Table 7.2. Experimental design for ethanolic fermentation with *S.cerevisiae* and *Z. mobilis*

Run	Microorganism	Broth
1	C1	SBMB3
2	C1	SBMB2
3	C2	SBMB0
4	C2	SBMB1
5	C2	SBMB3
6	C1	SBMB1
7	C1	SBMB0
8	C2	SBMB2
9	C1	SBMB2
10	C2	SBMB2
11	C1	SBMB4
12	C1	SBMB3
13	C2	SBMB3
14	C1	SBMB0
15	C2	SBMB4
16	C1	SBMB4
17	C2	SBMB1
18	C1	SBMB1
19	C2	SBMB0
20	C2	SBMB4

C1: *S. cerevisiae* C2: *Z. mobilis*, SBMB0, SBMB1, SBMB2, SBMB3, and SBMB 4: Hydrolyzates with the highest fermentable sugar content from treatments with 0, 0.5, 1.25, and 2.0% H_2SO_4, respectively at 135ºC and 45 min. SBMB4 is from the treatment T2C2t2 (120ºC, 1.25% H_2SO_4, 30 min.).

C. Results and Discussion

1. Tolerance of *S. cerevisiae* and *Z. mobilis* to inhibitory compounds

Furfural, HMF, acetic acid and their mixes produced cell growth inhibition on both *S. cerevisiae* and *Z. mobilis* when these strains were cultured in YM broth for 24 hours at 30ºC for most inhibitor concentrations. The minimum inhibitory concentration (MIC) for *S. cerevisiae* was 5 g/L of 5-HMF, 4 g/L of furfural, 10 g/L of acetic acid and the mix of 4/4/8 g/L of 5-HMF/furfural/acetic acid (Table 7.3). Most of these inhibitor concentrations for *S. cerevisiae* are in the ranges reported by Keatin et al., 2006; Taherzadeh, et al.,2000, Petersson et al., 2006; Larsson et al., 1999; Sanchez and Bautista (1998); and Mariorella, et al., 1983. The reduction in sugar consumption when these inhibitors were present can be attributed to the inhibition of glycolytic enzymes (Banerjee et al., 1981; Boyer et al., 1992). Keatin et al., 2006 reported that ethanol productivity in *S. cerevisiae* Y-1528 was markedly lower at the highest concentration of furfural (1.6 g/L) and 5-HMF (4 g/L).

Z. mobilis had a MIC of 3 g/L of 5-HMF, 2 g/L of furfural, 2 g/L of acetic acid and, a mix of 1/1/2 g/L 5-HMF/furfural/ acetic acid, which are in the ranges reported byDelgenes et al., (1996). When comparing both cells, 5-HMF showed less toxicity than furfural. Acetic acid had a higher toxic effect over *Z. mobilis* than *S. cerevisiae*, which was also confirmed by other authors (Wang, 2008, Lawford and Rousseau 2002, Mohagheghi et al. 2002, Kim et al., 2000) who found that *Z. mobilis* has low tolerance to acetic acid, which is commonly found in biomass hydrolyzates. Normally, trace minerals or metals are transported across the bacterial cell membrane by either active or passive mechanisms causing growth inhibition (Klinke et al. 2004). No evidence about the effect of all these mixed inhibitors in this cell was found in other studies,

which is important due to the synergistic inhibition effect that these compounds could have on the microorganisms.

When *S. cerevisiae and Z. mobilis* were cultivated in the detoxified SBM hydrolyzate, inhibitory effects were not shown in most cases, but *Z. mobilis* growth was totally inhibited (no growth at all) by the SBMB3 (135ºC, 2% H_2SO_4, 45 min.) and partially inhibited (slowed growth) by the SBMB2 (135ºC, 1.25% H_2SO_4, 45 min.). This inhibitory effect could be the result of salts and acetic acid (Table 7.4) still present in these broths due to the low effectiveness of the activated carbon to trap the salt of acetic acid, and the low salt-tolerance that *Z. mobilis* possess, which has been reported by several authors (Lawford and Rousseau 2002; and Mohagheghi et al. 2002, Doran, 1997).

Table 7.3. Tolerance of *S. cerevisiae* (NRRL Y-2233) to inhibitory compounds

Tube #	Concentrations of inhibitors (g/L) – (Results of growth)							
	5-HMF		**Furfural**		**Acetic acid**		**Inhibitors mix (5-HMF/Furfural/Acetic acid)**	
1	0	+	0	+	0	+	0/0/0	+
2	0.1	+	0.1	+	1	+	0.1/0.2/0.2	+
3	0.2	+	0.2	+	2	+	0.2/0.3/0.4	+
4	1	+	1	+	3	+	1/1/2	+
5	2	+	2	+	4	+	2/2/4	+
6	3	+	3	+	6	+	3/3/6	+
7	4	+	4	-	8	+	4/4/8	-
8	5	-	5	-	10	-	5/5/10	-
9	6	-	6	-	11	-	6/6/12	-
10	7	-	7	-	12	-	7/7/14	-
11	SBMB0						≈0/≈0/0.20	+
12	SBMB1						≈0/≈0/0.16	+
13	SBMB2						≈0/≈0/0.51	+
14	SBMB3						≈0/0.1/0.75	+
15	SBMB4						≈0/≈0/0.23	+

SBMB0 (135ºC, 0% H_2SO_4, 45 min.); SBMB1 (135ºC, 0.5% H_2SO_4, 45 min.); SBMB2 (135ºC, 1.25% H_2SO_4, 45 min.); SBMB3 (135ºC, 2% H_2SO_4, 45 min.); SBMB4 (120ºC, 1.25% H_2SO_4, 30 min.)

Table 7.4. Tolerance of *Z. mobilis* (NRRL B-4286) to inhibitory compounds

Tube #	Concentrations of inhibitors (g/L) – (Results of growth)							
	5-HMF		**Furfural**		**Acetic acid**		**Inhibitors mix (5-HMF/Furfural/Acetic acid)**	
1	0	+	0	+	0	+	0/0/0	+
2	0.1	+	0.1	+	1	+	0.1/0.2/0.2	+
3	0.2	+	0.2	+	2	-	0.2/0.3/0.4	+/-
4	1	+	1	+	3	-	1/1/2	-
5	2	+	2	-	4	-	2/2/4	-
6	3	-	3	-	6	-	3/3/6	-
7	4	-	4	-	8	-	4/4/8	-
8	5	-	5	-	10	-	5/5/10	-
9	6	-	6	-	11	-	6/6/12	-
10	7	-	7	-	12	-	7/7/14	-
11	SBMB0						≈0/≈0/0.20	+
12	SBMB1						≈0/≈0/0.16	+
13	SBMB2						≈0/≈0/0.51	+/-
14	SBMB3						≈0/0.1/0.75	-
15	SBMB4						≈0/≈0/0.23	+

SBMB0 (135ºC, 0% H_2SO_4, 45 min.); SBMB1 (135ºC, 0.5% H_2SO_4, 45 min.); SBMB2 (135ºC, 1.25% H_2SO_4, 45 min.); SBMB3 (135ºC, 2% H_2SO_4, 45 min.); SBMB4 (120ºC, 1.25% H_2SO_4, 30 min.)

2. Ethanol fermentation with *S. cerevisiae* and *Z. mobilis*

S. cerevisiae exhibited a good performance on the production of ethanol, sugars consumption, and cell growth in all the SBM broths used in this research (Figure 7.1). On the other hand, *Z. mobilis* was not as efficient in most of the fermentation broth used (Figure 7.2), especially with the SBMB3 (135ºC, 2%H_2SO_4, 45 min.) in which the bacteria did not grow at all.

It is highly likely that *Z. mobilis* was affected by inhibitory levels of salts coming from the SBM and sodium sulfate (Na_2SO_4) formed during the neutralization of the sulfuric acid with sodium hydroxide. Previous research showed that sodium acetate has serious inhibitory effects on the *Z. mobilis* growth (Yang et al., 2010a) who demonstrated that sodium acetate had more inhibitory effect than potassium and ammonium acetate on *Z. mobilis* and the combination of elevated Na+ and acetate ions causes a synergistic inhibitory effect for strain ZM4.

Kinetics of substrate consumption during the ethanol fermentation of hydrolyzed SBM broths

S. cerevisiae had its most rapid sugar consumption of 15.6 g/L during the first 12 hours of fermentation with the SBMB4 (120ºC, 1.25%H_2SO_4, 30 min.) and 15.1 g/L during the first 12 hours of fermentation with the SBMB1 (135ºC, 0.5%H_2SO_4, 45 min.) (Figure 7.1e). Therefore, the SBM broths with highest severity treatment during the hydrolysis led to a low rate of sugars consumption by *S. cerevisiae*, possibly due to the inhibitory effect of unknown toxic compounds remaining after the treatment and non-adsorbed during the detoxification.

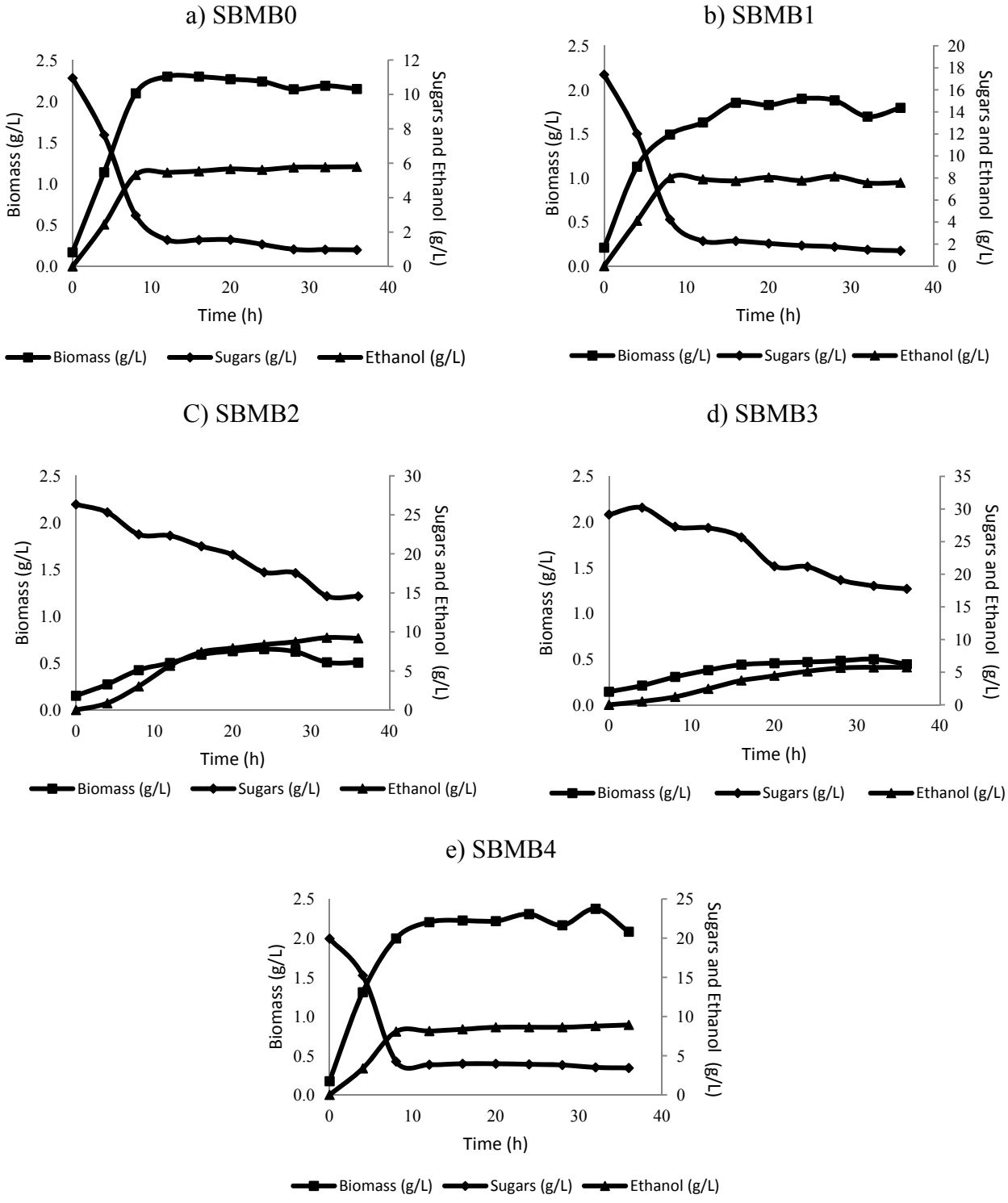


Figure 7.1. Fermentation profiles of SBM broths with *S. cerevisiae* (NRRL Y-2233). Acronyms explained at the bottom of next page

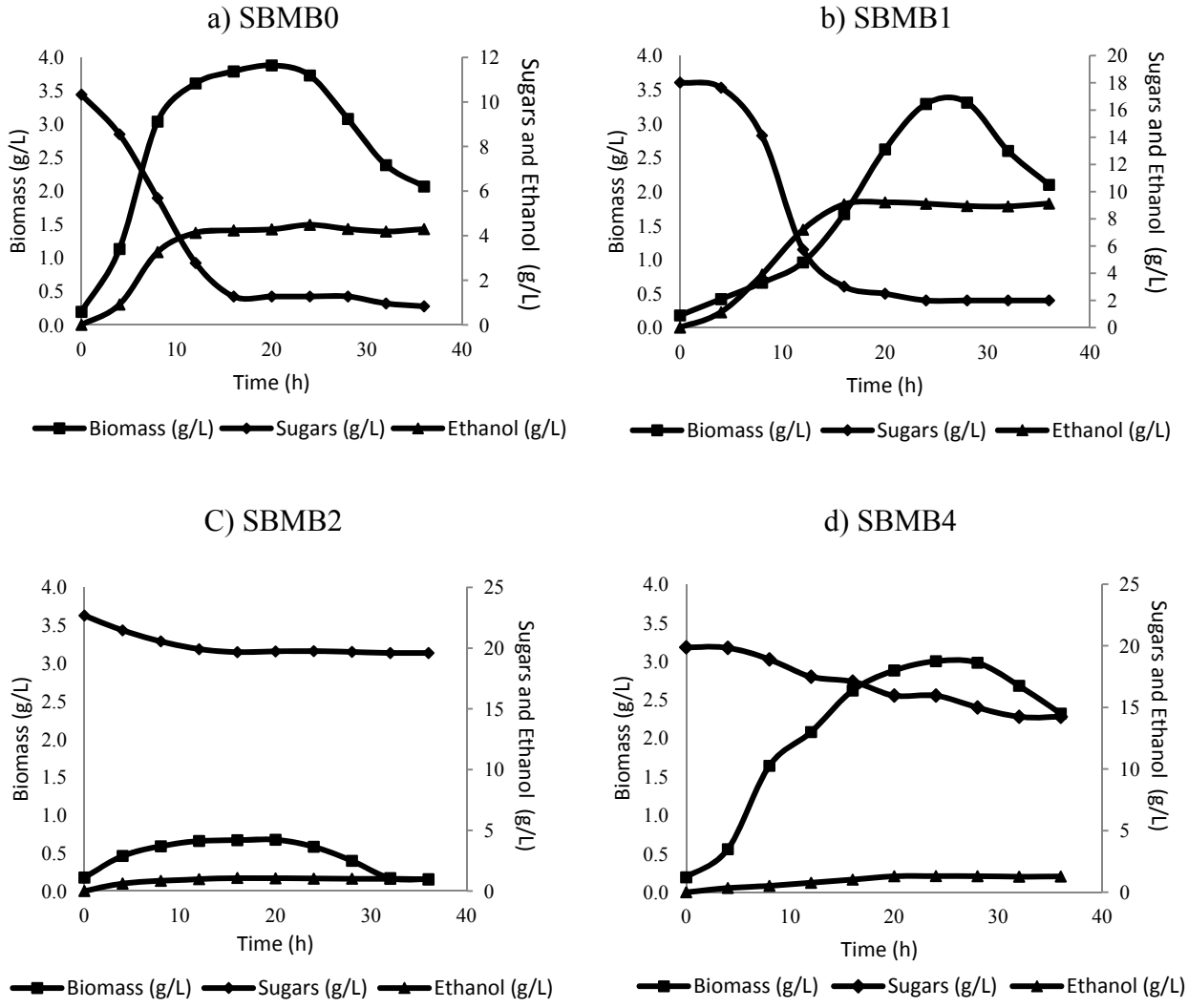


Figure 7.2. Fermentation profiles of SBM broths with *Z. mobilis* (NRRL B-4286).

SBMB0 (135ºC, 0% H_2SO_4, 45 min.); SBMB1 (135ºC, 0.5% H_2SO_4, 45 min.); SBMB2 (135ºC, 1.25% H_2SO_4, 45 min); SBMB3 (135ºC, 2% H_2SO_4, 45 min); SBMB4 (120ºC, 1.25% H_2SO_4, 30 min).

Z. mobilis also had its most rapid sugar consumption of 15.0 g/L per 16 hours of fermentation with the SBMB1 (135ºC, 0.5%H_2SO_4, 45 min.) (Figure 7.2b). As *S. cerevisiae*, *Z. mobilis* had low rates of sugar consumption in SBM broths produced at the highest severity during the pretreatments, especially SBMB2 (135ºC, 1.25%H_2SO_4, 45 min.), which is probably due to the inhibitory effect of acetic acid and salts as was discussed previously.

Overall, the maintenance coefficients m_s (g sugar consumed / g cell h) for both microorganisms did not have statistical significant differences except for *S. cerevisiae* with SBMB2 and SBMB3, which had higher values (2.7 and 2.8 g sugar/ g cell h, respectively) than the other coefficients (Table 7.5). However, numerically, all the ms coefficients for *Z. mobilis* were lower than the m_s coefficients for *S. cerevisiae* which means that the bacteria needs less carbon sources per cell than the yeast as it was reported by Dien, et al., 2003. Therefore, as it was established in Chapter 2, *Z. mobilis* needs less substrate per gram of cell produced than *S. cerevisiae* and has more carbon available for the production of ethanol (Dien et al., 2003).

Kinetics of ethanol production during fermentation of hydrolyzed SBM broths

S. cerevisiae had its maximum ethanol production of 8 g/L during the first 8 hours of fermentation of the SBMB1 (135ºC, 0.5%H_2SO_4, 45 min.) and SBMB4 (120ºC, 1.25%H_2SO_4, 30 min.). In contrast, the lowest ethanol production (5.67 g/L ethanol) was reached with the SBMB3 (135ºC, 2% H_2SO_4, 45 min.) after 28 hours of fermentation, where clearly some inhibitor compounds (salts, acetate from acetic acid, and other un-known toxic substances) could have reduced the ethanol productivity.

Ethanol production by *Z. mobilis* peaked at 9.2 g/L ethanol after 20 hours of fermentation with the SBMB1 (135ºC, 0.5% H_2SO_4, 45 min.). The SBMB0 (135ºC, 0% H_2SO_4, 45 min.) allowed a maximum ethanol rate of 3.9 g/L ethanol after 20 hours of fermentation. On the other

hand, the lowest ethanol productions—1 g/L at 20 h—were attained with the SBMB2 (135ºC, 1.25% H_2SO_4, 45 min.) and SBMB4 (120ºC, 1.25% H_2SO_4, 30 min.), which is likely caused by inhibitors (acetate from acetic acid, other salts, and unknown compounds). There is evidence that ethanol production with *Z. mobilis* and its growth can be reduced importantly under low concentrations (2 to 8 g/L) of acetic acid (Wang, Y. 2008).

The ethanol yield (Yp/s, g ethanol/ g of sugars) reported in Table 5 did not show significant statistical differences between the two microorganism and SBM broths used. However, numerically both microorganisms showed similar ethanol yield to the theoretical values, which is 100% for 0.51 g ethanol/ g of sugars (Doran, 1997). Fermentations in the SBM0 and SBMB1 gave the highest ethanol yields, 96% of the theoretical for both microorganisms, which are comparable or even higher with values reported in other researches (Letti, et al., 2012, Romao, et al., 2012; Da Cunha-Pereira, et al., 2011; Zhang and Feng, 2010; Siqueira, et al., 2008) . Additionally, the maximum ethanol volumetric productivity (rp) for *S. cerevisiae* was reached with the SBM1 (1.01g ethanol/L h) and SBMB4 (1.00 g ethanol/L h), which did not have significant differences among them, but it had differences with respect to the other broths used in this research (Table 7.5).

Table 7.5. Means of kinetic parameters of ethanol fermentation in batch culture with *S. cerevisiae* (NRRL Y-2233) and *Z. mobilis* subspecies *mobilis* (NRRL B-4286) in hydrolyzed soybean meal broths ± SE

SBM broth	MO	Yp/s (g ethanol/g sugar)	Yx/s (g cell/g sugar)	ms (g sugar/g cell h)	rp (g ethanol/Lh)
SBMB0	*S. cerevisiae*	0.49±0.021 a	0.23±0.014 abc	0.69 b	0.67±0.028 b
SBMB0	*Z. mobilis*	0.49±0.007 a	0.23±0.057 ab	0.44 b	0.37±0.007 cd
SBMB1	*S. cerevisiae*	0.49±0.014 a	0.10±0.014 cd	1.19 b	1.00±0.028 a
SBMB1	*Z. mobilis*	0.48±0.028 a	0.08±0.014 d	0.97 b	0.61±0.007 bc
SBMB2	*S. cerevisiae*	0.46±0.007 a	0.04±0.007 d	2.73 a	0.61±0.113 bc
SBMB2	*Z. mobilis*	0.26±0.042 a	0.15±0.021 bcd	0.65 b	0.11±0.007 de
SBMB3	*S. cerevisiae*	0.40±0.057 a	0.02±0.007 d	2.78 a	0.29±0.092 de
SBMB4	*S. cerevisiae*	0.49±0.007 a	0.11±0.007 bcd	1.13 b	1.01±0.021 a
SBMB4	*Z. mobilis*	0.29±0.113 a	0.32±0.014 a	0.17 b	0.07±0.007 e

SBMB0 (135ºC, 0% H_2SO_4, 45 min.); SBMB1 (135ºC, 0.5% H_2SO_4, 45 min.); SBMB2 (135ºC, 1.25% H_2SO_4, 45 min.); SBMB3 (135ºC, 2% H_2SO_4, 45 min.); SBMB4 (120ºC, 1.25% H_2SO_4, 30 min.). Means followed by the same letters in each column did not have significant differences. For examples of calculations, see Appendix III.

These values for *S. cerevisiae* are comparable and some of them, even, higher than the reported for other researchers (Da Cunha-Pereira, et al., 2011) On the other hand, *Z. mobilis* only reached a maximum ethanol volumetric productivity (rp) of 0.61 g ethanol/Lh also with the SBM1, which is lower than the values, 1.4 to 1.8 g ethanol/Lh, reported by Letti, et al. (2012) with *Z. mobilis* during the production of ethanol using SBM molasses. Also, it is lower than the values (2.8 gethanol/Lh) reported by Zhang and Feng (2010) during the production of ethanol with *Z. mobilis* from sweet potato; however is higher than 0.59 g/Lh obtained by Mohagheghi et al. (2002) using lignocellulosic material as substrate. These results demonstrate that the application of *Z. mobilis* in the ethanol fermentation is not feasible when the substrate is SBM broth obtained under the conditions applied in these experiments. This could be explained by the high concentration of acetic acid, salts and other toxic unknown compounds formed during the acid hydrolysis in the broths obtained under severe treatments.

Biomass growth kinetics and model development for the ethanolic fermentation

Data reported in Figure 7.1a show that *S. cerevisiae* had its maximum biomass concentration (2.3 g/L) after 12 hours of anaerobic fermentation when using the SBM0 (135ºC, 0% H_2SO_4, 45 min, followed by enzymatic treatment with cellulase. Also, this maximum concentration of biomass (2.3 g/L) was reached with SBMB4 (120ºC, 1.25% H_2SO_4, 30 min.) at 24 hours. This difference in time could have been the result of the former substrate having more inhibitors and salt contents since the acid concentration for the pretreatment was higher than the latter. On the other hand, SBMB3 (135ºC, 2% H_2SO_4, 45 min.) exhibited the major inhibition over the yeast growth since the biomass reached not more than 0.5 g/L after 32 hours of fermentation. Overall, production of biomass by *S. cerevisiae* showed a correlation with ethanol production.

Z. mobilis had its highest biomass concentration, 3.9 g/L, after 20 hours of fermentation (Figure 7.2a) with the SBMB0 (135ºC, 0%, H_2SO_4, 45 min., followed by enzymatic treatment with cellulase. Likewise, the biomass produced (3.3 g/L) when fermenting the SBMB1 (135ºC, 0.5% H_2SO_4, 45 min) was very close to the former but was reached in 24 hours. In contrast, SBM2 (135ºC, 2% H_2SO_4, 45 min.) exhibited the major inhibitory effects over the *Z. mobilis* growth probably due to this broth containing higher concentrations of salts, acetic acid and other inhibitors that restricted its growth (Yang et al., 2010a; Doran, 1997). In general, *Z. mobilis* also showed a growth-associated ethanol production with the exception of those broths were the bacteria were considerably inhibited.

Data of cell growth fitted the logistic model during the first 24 hours of fermentation and are shown in Table 7.6 and Appendices 1 and 2. The maximum specific growth rates (μ_{max}) shows that in the SBM0 and SBMB4, μ_{max} for *S. cerevisiae* (0.63and 0.60 h^{-1}, respectively) were higher but not significant different than μ_{max} for *Z. mobilis*, 0.55 h^{-1}, in the substrate T3C0t3. Using also the logistic model, Wang et al. (2004) reported a μ_{max} of 0.1 h^{-1} for the ethanolic fermentation of apple juice with *S. cerevisiae* and Huang and Wang (2010) obtained a μ_{max} of 5.2 h^{-1} using *Saccharomyces diastaticus* and mixed sugars as a substrate. For *Z. mobilis,* Mohagheghi et al. (2002) reported a μ_{max} of 0.34 h^{-1} for in the ethanolic fermentation of hydrolyzed lignocellulosic biomass. On the whole, μ_{max} for *S. cerevisiae* was higher for substrates obtained with pretreatments of less severity; however in the case of *Z. mobilis* μ_{max} did not show the same pattern due to the effect of inhibitors present in some the SBM broths.

The initial concentration of biomass (X_0) was not significantly different among all the broths for both microorganisms, which is an indication that the inoculum was homogeneously prepared and inoculated to each SBM broth. The other kinetic parameter obtained by fitting the

logistic model among the first 24 hours of fermentation was the maximum concentration of biomass (Xm). For *S. cerevisiae*, Xm was 2.28 and 2.23 g/L for SBMB0 and SBMB4 respectively, while the maximum Xm for *Z. mobilis* was 3.79 g/L and 5.1 g/L for the SBMB0 and SBMB1, respectively.

The biomass yield from substrate (Yx/s) showed in Table 7.5 was significantly higher for *S. cerevisiae* (0.1 g cell/ g sugar consumed) than *Z. mobilis* (0.08 g cell/ g sugar consumed) when fermenting the SBMB1. Higher Yx/s values with *S. cerevisiae* than *Z. mobilis* were also reported by other researchers (Dien, et al., 2003, Aitabdelkader and Baratti, 1993). This can be explained for the better resistance of the yeast than the bacteria to the inhibitors present in this substrates. However, with SBM2 the relation was the opposite, Yx/s value for *Z. mobilis* was 0.15 g cell/g sugar and for *S. cerevisiae* was 0.04 g cell / g sugar. Nonetheless, most of the Yx/s values for *S. cerevisiae* were higher than the reports by Siqueira, et al. (2008) in the production of bio-ethanol from SBM molasses or even similar values (0.14 g/g) as it was reported by Ahmad, et al. (2011)

Table 7.6. Means of logistic model parameters of ethanol fermentation in batch culture with *S. cerevisiae* (NRRL Y-2233) and *Z. mobilis* subspecies *mobilis* (NRRL B-4286) in hydrolyzed soybean meal broths ± SE

SBM broth	**Microorganism**	μ_{max}	X_0	**Xm**
SBMB0	*S. cerevisiae*	0.63±0.12 a	0.17±0.08 a	2.28±0.05 a
SBMB0	*Z. mobilis*	0.55±0.05 a	0.18±0.05 a	3.79±0.06 f l
SBMB1	*S. cerevisiae*	0.45±0.09 afgh	0.32±0.09 a	1.81±0.05 b
SBMB1	*Z. mobilis*	0.16±0.02 bk	0.18± 0.04a	5.1±0.92 g l
SBMB2	*S. cerevisiae*	0.21±0.11 bdfi	0.16±0.08 a	0.66±0.10 cej
SBMB2	*Z. mobilis*	0.45±0.25 aijkl	0.18±0.1 a	0.65±0.05 hjk
SBMB3	*S. cerevisiae*	0.19±0.15 begj	0.14±0.08 a	0.49±0.12 dek
SBMB4	*S. cerevisiae*	0.60±0.11 a	0.24±0.09 a	2.23±0.05 a
SBMB4	*Z. mobilis*	0.29±0.03 cdehl	0.26±0.06 a	2.97±0.09 i

SBMB0 (135ºC, 0% H_2SO_4, 45 min.); SBMB1 (135ºC, 0.5% H_2SO_4, 45 min.); SBMB2 (135ºC, 1.25% H_2SO_4, 45 min.); SBMB3 (135ºC, 2% H_2SO_4, 45 min.); SBMB4 (120ºC, 1.25% H_2SO_4, 30 min.). Means followed by the same letters in each column did not have significant differences.

D. Conclusions

The minimum inhibitory concentration (MIC) for *S. cerevisiae* (NRRL Y-2233) was 5 g/L 5-HMF, 4 g/L furfural, 10 g/L acetic acid and a mix of 4/4/8 g/L of 5-HMF/furfural/acetic acid; while, *Z. mobilis* had a MIC of 3 g/L 5-HMF, 2 g/L furfural, 2 g/L acetic acid and a mix of 1/1/2 g/L of5-HMF/furfural/ acetic acid. Furthermore, inhibitory effects were not demonstrated in the majority of the cases when *S. cerevisiae* and *Z. mobilis* were cultivated in the hydrolyzed SBM broth previously detoxified with activated carbon, but the bacteria's growth was inhibited by the SBM3 (135ºC, 2% H_2SO_4, 45 min.) and partially attenuated by the SBM2 (135ºC, 1.25% H_2SO_4, 45 min.).

S. cerevisiae exhibited a good performance in the production of ethanol, sugars consumption, and cell growth in all the SBM broths used in this research; on the other hand, *Z. mobilis* did not yield good profiles in most of the fermentation broth used, especially with the SBM3 (135ºC, 2%H_2SO_4, 45 min.) in which the bacteria did not growth. Growth data fitted the logistic model satisfactorily for both microorganisms in the calculation of the kinetic parameters μ_{max}, X_{max}, and X_0.

The maximum ethanol production (8 g/L ethanol) of *S. cerevisiae* was reached during the first 8 hours of fermentation with the SBM1 (135ºC, 0.5%H_2SO_4, 45 min.) and SBMB4 (120ºC, 1.25%H_2SO_4, 30 min.). On the other hand, *Z. mobilis* yielded maximum ethanol production (9.2 g/L ethanol) during the first 20 hours of fermentation also with the SBM1(135ºC, 0.5% H_2SO_4, 45 min.).

CHAPTER VIII

Conclusions

This research demonstrated that the dilute acid hydrolysis of SBM is a suitable method to produce fermentable sugars for bioethanol production and other important products in the food industry, such as lactic, citric or acetic acid, xylitol, butanol, or even microbial biomass. Also, most treatments applied in this research enhanced considerably the crude protein content of this material which is used in the animal feed industry. The liquid fraction of SBM after dilute acid hydrolysis reached 21% d.b. fermentable sugars when was treated at 80ºC with 1.9-2% H_2SO_4 for 7-16 h. This treatment also reduced the amount of stachyose and raffinose and produced low concentrations of 5-HMF (less than 0.21 g/L). The crude protein content in the SBM solid fraction increased from 48 t0 58% d.b. after treatments ranging from 0.25 to 1.7% of H_2SO_4 for 0.5 to 2.5 h at 80 ºC, while the color was similar to the untreated meal, which was a premise of this research.

When SBM was treated at temperatures above 100^{o}C in combination with dilute acid, the treatment 135°C, 2% H_2SO_4, and 45 min (T3C3t3), produced 32.2% d.b. of fermentable sugars which represent 11 percentage points increase from the treatments at 80°C, which is likely due to these more severe conditions degrading part of the cellulose, hemicellulose, and other polysaccharides structures into sugars. However, these conditions did not yield the maximum amount of crude protein. The treatment T2C1t3 (120°C, 0.5% H_2SO_4, and 45 min) increased the protein content from 48.1% d.b. to 58.6% d.b with no significant color change when compared with the untreated meal. A good and important balance in the SBM liquid fraction between high fermentable sugars (16.2% d.b.), crude protein (55.5% d.b.) and color (solid fraction) was reached with the treatment T2C2t2 (120°C, 1.5% H_2SO_4, and 30 min). Additionally; the level of

5-HMF and furfural (0.0018 g/L and 0.32 g/L, respectively) at temperatures above 100°C were not highly toxic for the microorganisms used; however, acetic acid reached a high concentration (0.9 g/L) under the treatment T3C3t3 (135°C, 2.0% H_2SO_4, and 45 min), which showed to cause inhibition and affected growth and ethanol production, especially for *Z. mobilis*, which is less resistant to this kind of toxic compounds.

Enzymatic treatments applied in the acid-hydrolyzed SBM had the best results when cellulase was used alone with the pretreated SBM at 135°C, 0% H_2SO_4, 45 min. Cellulase applied to this substrate reached the highest increment in fermentable sugars (12.34 g/L), which was no higher than the acid treatments. In contrast, substrates that were treated with high temperature, high acid concentration, and longer time did not benefit much from the enzymatic treatment; because the majority of polysaccharides were already hydrolyzed by the acid treatment and higher content of toxic compounds were present, which inhibited the enzymes. Also, the concentrations of inhibitors in these treatments were high even after detoxification which reduced significantly 5-HMF and furfural concentrations, but did not decrease significant levels of acetic acid. Less than 1% of fermentable sugars were loosed after the detoxification treatments and the maximum reductions in 5-HMF and furfural were 90.20 ± 1.01% (mean ± S.E.) and 96.75 ± 0.85%, respectively.

The liquid fraction of the SBM after the acid and enzymatic treatments contained ammonium-Nitrogen (NH_4^+-N) at concentrations between 0.20 to 1.24 g/L, which are high enough to be a source of nitrogen for fermentations. In the solid fraction the lysine bioavailability increased considerably after all the treatments applied in this research. The most successful hydrolysis treatment at 135°C, 45 min., 0% H_2SO_4 plus mix of cellulose + ß-glucosidase increased lysine bioavailability from 82% to 97% d.b. However, significant

differences were not detected among the treatments which can be interpreted as an advantage because it would be possible to obtain improvement in lysine bioavailability under less severe treatments even without enzymatic treatments which can make this process more profitable.

The minimum inhibitory concentration (MIC) for *S. cerevisiae* was 5 g/L of 5-HMF, 4 g/L of furfural, 10 g/L of acetic acid and a mixture of 4/4/8 g/L (5-HMF/furfural/acetic acid); while for *Z. mobilis* the MIC was 3 g/L of 5-HMF, 2 g/L of furfural, 2 g/L of acetic acid and a mix of 1/1/2 g/L (5-HMF/furfural/ acetic acid). When *S. cerevisiae and Z. mobilis* were grown in hydrolyzed SBM broth after detoxification with activated carbon, inhibitory compounds did not have an effect in the majority of the cases. However, the bacteria did not growth in hydrolyzates obtained at 135ºC, 2% H_2SO_4, 45 min (SBMB3) and grew only partially in the hydrolyzate obtained at 135ºC, 1.25% H_2SO_4, 45 min (SBMB2), which indicates that *Z. mobilis* is affected by some toxic compounds such as salts, acetate from acetic acid, and other unknown inhibitors generated under these condition.

During the batch fermentation, *S. cerevisiae* had its maximum bioethanol production (8 g/L bioethanol) during the first 8 hours when using the soybean meal broth obtained at 135ºC, 0.5% H_2SO_4, 45 min. and at 120ºC, 1.25% H_2SO_4, 30 min. However, *Z. mobilis* had its maximum bioethanol production (9.2 g/L bioethanol) during the first 20 hours of fermentation also with the broth obtained at135ºC, 0.5% H_2SO_4, 45 min. Overall, due to the high levels of nutrients, carbon and nitrogen sources, *S. cerevisiae* exhibited a good performance in the production of bioethanol, sugars consumption, and cell growth in all the SBM broths used in this research; however, *Z. mobilis* did not yield good profiles in most of the fermentation broth used in this research, especially with the broth obtained at 135ºC, 2%H_2SO_4, 45 min. in which the

bacteria did not grow due to the high levels of toxic compounds (acetate from acetic acid and other salts) present in these medium.

This study showed the feasibility of extracting fermentable sugars from soybean meal by acid treatment with sulfuric acid at low concentrations and enzymatic treatment, which also increased the protein content and bioavailability in the solid phase. Further studies in this line of research should explore other hydrolysis methods, such as chemical or physical, and substances (acids, bases, or water alone) under different conditions of temperature and time in order to achieve better yields of fermentable sugars and less toxic compounds. Additionally, further studies could focus in determining if additional toxic compounds are present that may cause inhibitory effects in the growth and bioethanol production of *Z. mobilis*. Furthermore, toxicological studies of the hydrolyzed soybean meal solid fraction will be necessary in order to demonstrate that this product will not be toxic as animal feed. Finally, studies in genetic modification must be done in order to develop more robust *Z. mobilis* strains capable to resist higher concentrations of inhibitors such as salts, acetic acid, 5-HMF, and furfural, and other potential toxic compounds formed during the acid hydrolysis of SBM.

CHAPTER IX

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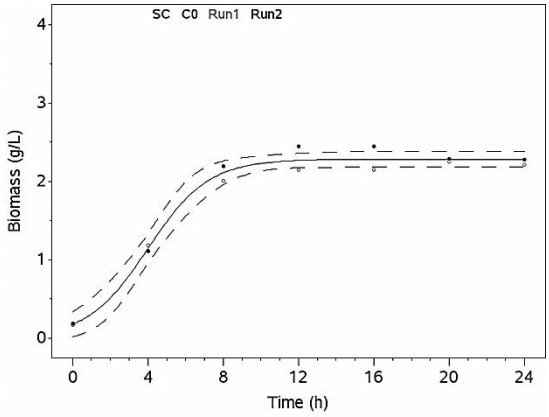
APPENDICES

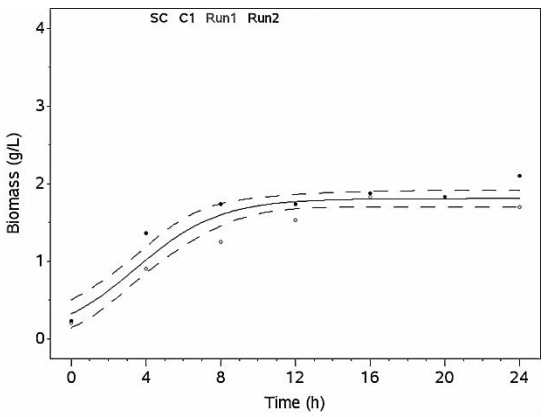
Appendix I

Biomass growth for *S. cerevisiae* (NRRL Y-2233) in hydrolyzed soybean meal broths obtained by fitting the logistic model with SAS. T3C0t3 (1355ºC, 0% H_2SO_4, 45 min.); T3C1t3 (135ºC, 0.5% H_2SO_4, 45 min.); T3C2t3 (135ºC, 1.25% H_2SO_4, 45 min.); T3C3t3 (135ºC, 2% H_2SO_4, 45 min.); T2C2t2 (120ºC, 1.25% H_2SO_4, 30 min.)

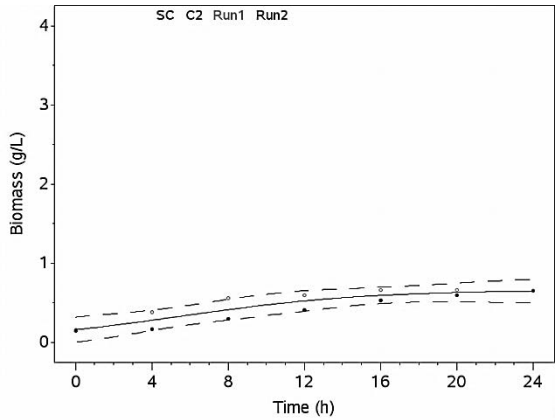
a) T3C0t3



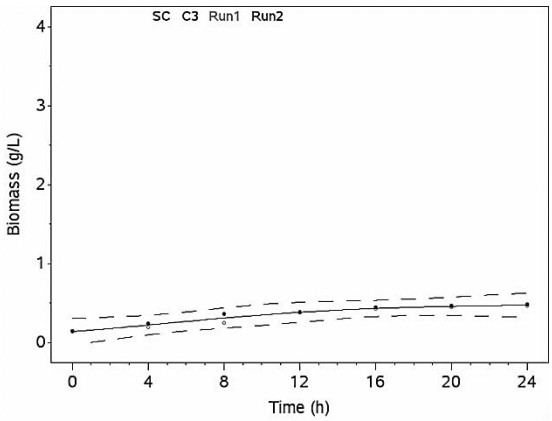
b) T3C1t3



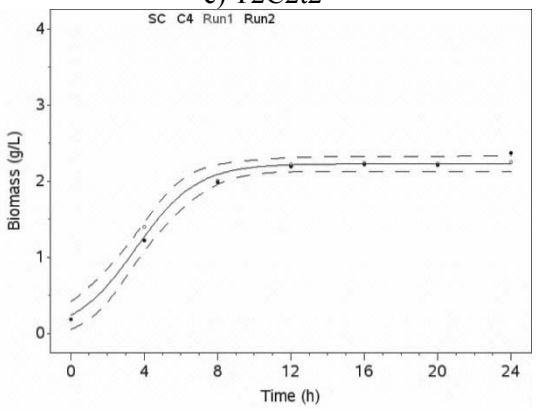
c) T3C2t3



d) T3C3t3



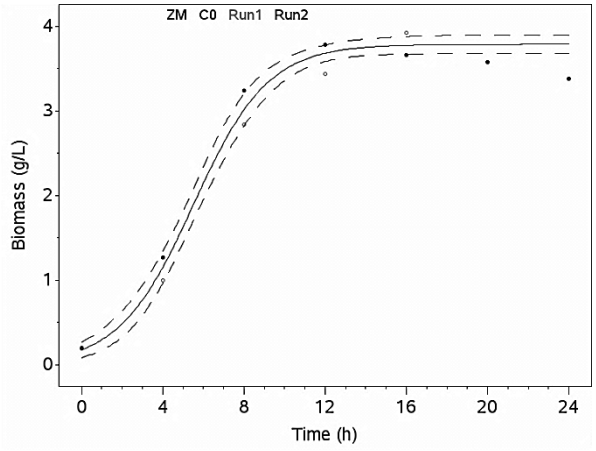
e) T2C2t2



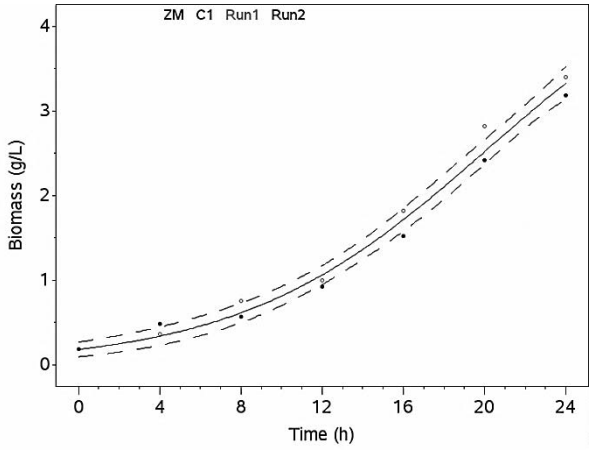
Appendix II

Biomass growth for *Zymomonas mobilis* subspecies mobilis (NRRL B-4286) in hydrolyzed soybean meal broths obtained by fitting the logistic model with SAS. T3C0t3 (135ºC, 0% H_2SO_4, 45 min.); T3C1t3 (135ºC, 0.5% H_2SO_4, 45 min.); T3C2t3 (135ºC, 1.25% H_2SO_4, 45 min.); T3C3t3 (135ºC, 2% H_2SO_4, 45 min.); T2C2t2 (120ºC, 1.25% H_2SO_4, 30 min.)

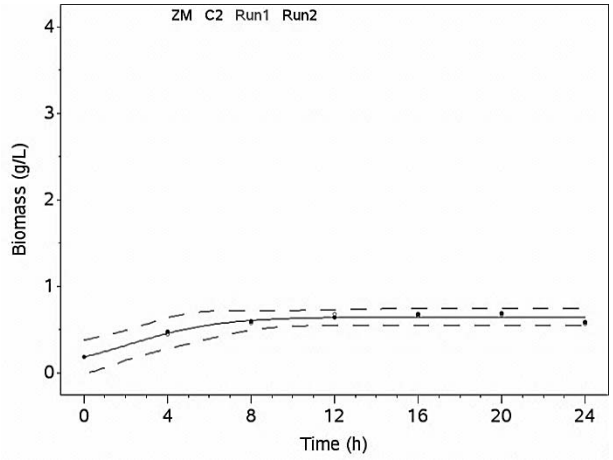
a) T3C0t3



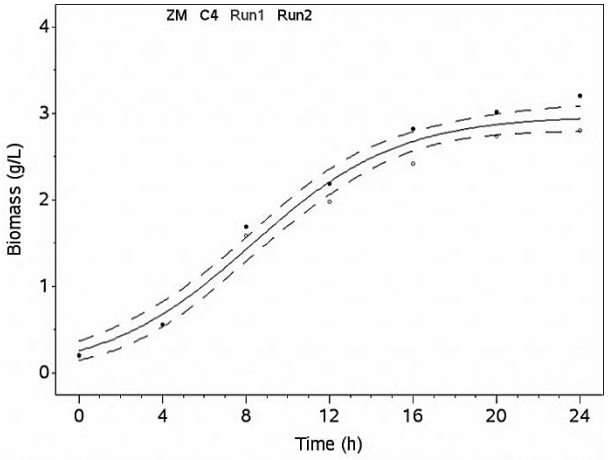
b) T3C1t3



c) T3C2t3



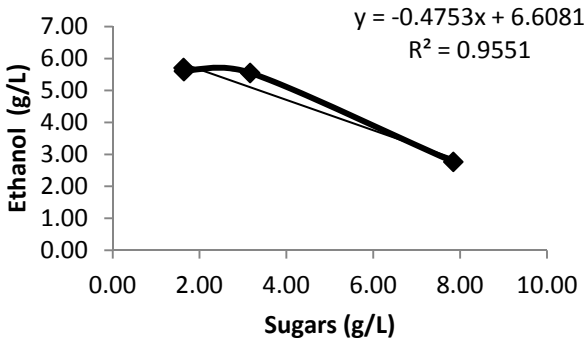
d) T2C2t2



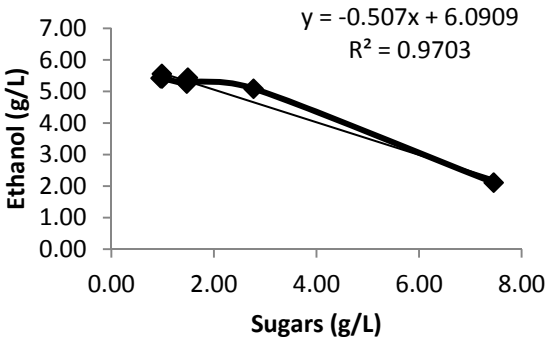
Appendix III

Examples of calculation for the kinetic parameters shown in Table 7.5

Example 1: Yp/s calculation for SBMB0



Repetition A



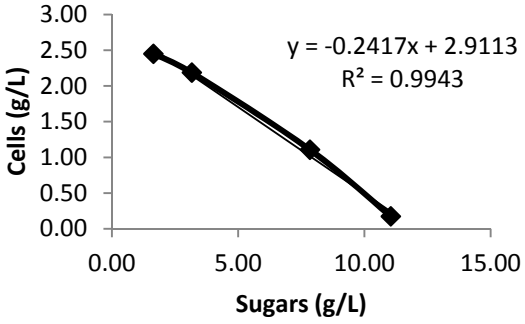
Repetition B

Yp/s for Repetition A = 0.48 g ethanol/ g sugar

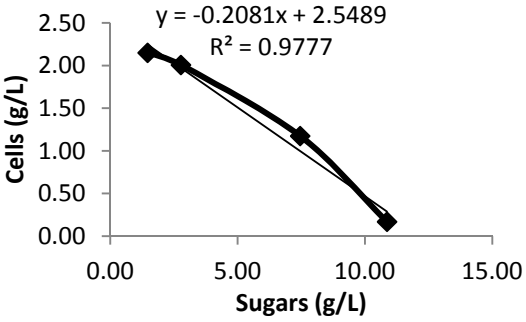
Yp/s for Repetition A = 0.51 g ethanol/ g sugar

Average Yp/s = 0.49 g ethanol/ g sugar

Example 2: Yx/s calculation for SBMB0



Repetition A



Repetition B

Yx/s for Repetition A = 0.24 g cell/ g sugar

Yx/s for Repetition A = 0.21 g cell/ g sugar

Average Yp/s = 0.23 g cell/ g sugar

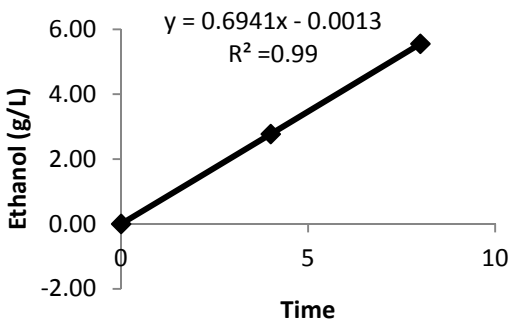
Example 3: Maintenance coefficient (ms) calculation for SBMB0

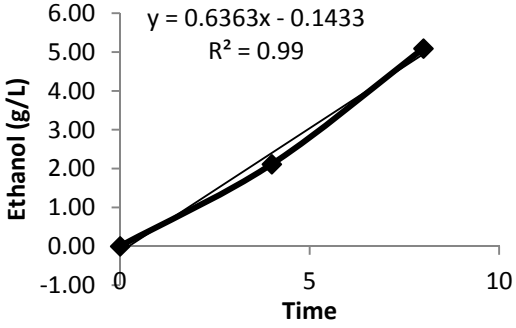
Maintenance coefficient (g sugar/g cell h) was calculated by using the equation 7.5:

$$ms = \frac{\left[(rs) - \frac{rx}{Yxs} - \frac{rp}{Yps}\right]}{x}$$

ms = -((0.82)-(0.25/0.24)-(0.69/0.47))/(2.45) = 0.69 (average)

Example 4: Volumetric ethanol productivity, rp (g ethanol/Lh) for SBMB0





Repetition A

Repetition B

rp for Repetition A = 0.69 g ethanol/ L h

rp for Repetition A = 0.64 g ethanol/ L h

Average rp = 0.67 g ethanol/ L h